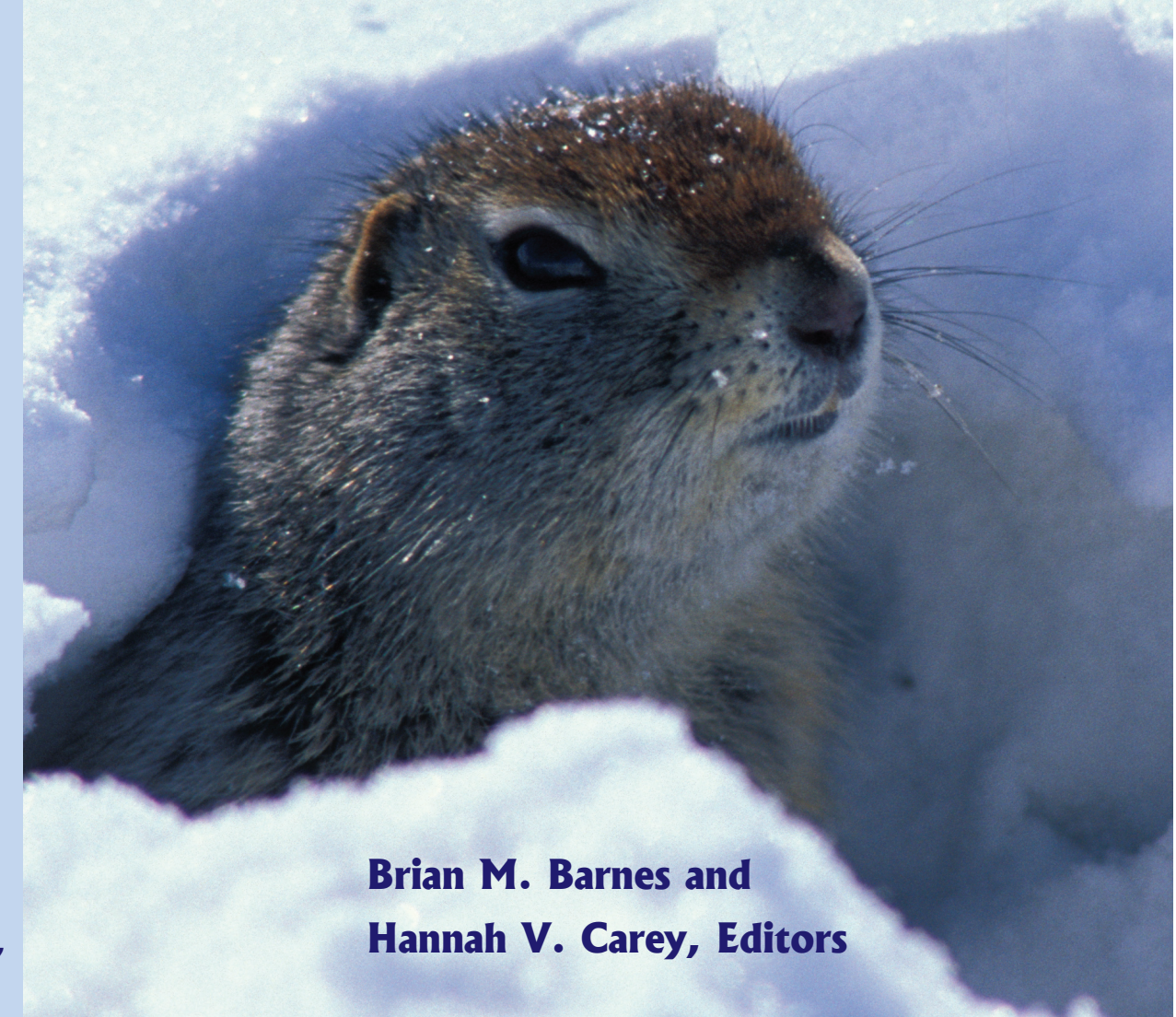




Life in the Cold

Evolution, Mechanisms, Adaptation, and Application



**Brian M. Barnes and
Hannah V. Carey, Editors**

Life in the Cold:

Evolution, Mechanisms, Adaptation, and Application

Barnes and Carey,
editors

Life in the Cold: Evolution, Mechanisms, Adaptation, Application compiles the latest results, reviews, and syntheses on strategies and mechanisms that insects, fish, amphibians, reptiles, mammals, and birds use for enduring anticipated famine, cold, and seasonality.

Especially covered is hibernation in mammals: its evolution, molecular basis, physiology, neurobiology, and potential for biomedical application of discoveries to the development of novel human therapies that mitigate stress and trauma.

These chapters are by scientists who attended the Twelfth International Hibernation Symposium, 2004, while sailing from Vancouver, British Columbia, to Seward, Alaska.

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emerges from its burrow in the spring.

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Preface

The year 2004 marks 45 years since the first international conference on biological adaptations to the cold was organized by Charles P. Lyman and Albert R. Dawe. This book holds contributions associated with the Twelfth International “Life in the Cold” symposium, which, since 1959, has been held every three to four years. This truly international symposium was held on board the MS *Veendam*, sailing from Vancouver, British Columbia, Canada, to Seward, Alaska, USA from 25 July to 1 August 2004. Since the last conference in Jungholz, Austria, in 2000, the organizers note with sadness the passing of C.P. Lyman and E.T. Pengelley, two eminent inventors and leaders in the fields of hibernation biology and circannual rhythms. This volume is dedicated to Charles Lyman and Ted Pengelley with respect for their contributions and much thanks for their mentorship to young scientists.

Life in the Cold 2004 includes disciplines outside of the traditional mammalian hibernation realm of the series. Entire sessions are devoted to overwintering strategies in amphibians and reptiles, in insects, and in birds. While the study animals vary, the questions and often the solutions that allow animals to persevere through seasons of anticipated famine and/or cold are similar; we hope this confluence of different “ologists”: mammal, ornithol, entomol, herpet, and ichthyol, will spawn collaborations, synergisms, and discovery. Also new to this conference is a session that focuses entirely on biomedical applications of hibernation and other overwintering strategies. The inclusion of applied biology reflects the growing appreciation by the scientific community of the potential value that understanding of the fundamental basis of hibernation and other adaptations to environmental extremes can provide in terms of innovative approaches to animal and human biomedicine. We expect this appreciation to continue to grow as our discipline attracts increasing numbers of scientists outside the traditional fields of zoology and behavior. Furthermore, with increased development and application of new analytic tools, the pace of understanding of the evolution, adaptations, and mechanisms that permit life in cold and extreme environments will quicken, and soon result in applications to saving human lives.

The editors give thanks for help from the scientific reviewers of each chapter, the session organizers and the contributors. We thank Sue Mitchell for book design, layout, and proof reading, Øivind Tøien for the cover photograph, and Shalen Bezona, Annie Scrivanich, and Alison York for assistance in tracking, planning, and organization. We gratefully acknowledge sponsorship by the Institute of Arctic Biology, University of Alaska Fairbanks, the National Science Foundation, Office of Polar Programs, and the Mini Mitter Company.

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The continuance of this series of conferences and books on investigations of biological adaptation to cold and seasonality depends on the initiative of present and future generations of enthusiasts. May this tradition continue!

—Brian M. Barnes, Fairbanks, Alaska

—Hannah V. Carey, Madison, Wisconsin

First and Corresponding Author Contact Information

Radoslav K. Andjus (Deceased)**Marina Marjanovic**

Eastern Illinois University
Department of Biological Sciences
Charleston, IL, 61920 USA
marinam@uiuc.edu

Denis V. Andrade

Departamento de Zoologia
Universidade Estadual Paulista
Av. 24-A, 1515
Rio Claro, São Paulo, 13506-900 Brasil
denis@rc.unesp.br

Matthew T. Andrews

University of Minnesota Duluth
Biology Department
211 Life Science
10 University Dr.
Duluth, MN 55812 USA
mandrews@d.umn.edu

Thomas Arendt

Paul Flechsig Institute of Brain Research
Department of Neuroanatomy
University of Leipzig
Jahnallee 59
D-04109 Leipzig, Germany
aret@medizin.uni-leipzig.de

Kenneth B. Armitage

University of Kansas
Ecology and Evolutionary Biology
1036 Haworth
Lawrence, KS 66045 USA
marmots@lark.cc.ukans.edu

Patrick J. Baker

Miami University
Department of Zoology
Oxford, OH 45056 USA
bakerpj@muohio.edu

B.M. Barnes

University of Alaska Fairbanks
Institute of Arctic Biology
311 Irving I, Box 757000
Fairbanks, AK 99775 USA
brian.barnes@uaf.edu

Philip E. Bickler

Department of Anesthesia
University of California at San Francisco
San Francisco, CA 94143-0542 USA
bicklerp@anesthesia.ucsf.edu

Claudia Bieber

Research Institute of Wildlife Ecology
University of Veterinary Medicine
Veterinärplatz 1
A-1210 Wien
Vienna, Austria
claudia.bieber@vu-wien.ac.at

Decheng Cai

National Institutes of Health
Branch National Institute of Neurological
Disorders and Stroke
10 Center Drive, MS1260
Bethesda, MD 20892-1260 USA

Hannah V. Carey

University of Wisconsin
 Department of Comparative Biosciences
 School of Veterinary Medicine
 4154 Veterinary Medicine Building
 2015 Linden Dr
 Madison, Wisconsin 53706 USA
 careyh@svm.vetmed.wisc.edu

Andrew R. Cossins

School of Biological Sciences
 University of Liverpool
 BSB 237 Biosciences Building
 Crown Street, Liverpool L69 7ZB, UK
 cossins@liverpool.ac.uk

Patricia J. DeCoursey

Department of Biological Sciences
 University of South Carolina
 Columbia, SC 29208 USA
 dcoursey@biol.sc.edu

Arthur L. DeVries

University of Illinois
 Department of Animal Biology
 524 Burrill Hall, MC 114
 407 S Goodwin
 Urbana/Champaign, IL 61801 USA
 adevries@uiuc.edu

Kelly L. Drew

Institute of Arctic Biology
 University of Alaska Fairbanks
 311 Irving I, Box 757000
 Fairbanks, AK 99775 USA
 ffkld@uaf.edu

John G. Duman

University of Notre Dame
 Department of Biological Sciences
 Notre Dame, IN 46556 USA
 John.G.Duman.1@nd.edu

L. Elaine Epperson

University of Colorado School of Medicine
 S/M Cellular & Structural Biology
 Biomedical Research Building, Room 702
 Campus Box B111
 Denver, CO 80301, USA
 elaine.epperson@uchsc.edu

Vadim V. Fedorov

Cardiology Research Center
 3 Cherepkovskaya 15a
 121552 Moscow, Russia
 fnvf@uaf.edu

Gregory L. Florant

Department of Biology
 Colorado State University
 E205 Anatomy-Zoology
 Fort Collins, CO 80523 USA
 gregory.florant@colostate.edu

Alan R. French

Department of Biology
 Penn State University
 SC-3 Hayfield Building
 Wilkes-Barre Campus
 Lehman, PA 18627, USA
 afrench@binghamton.edu

Craig L. Frank

Fordham University
 Louis Calder Center
 53 Whippoorwill Rd., Box K
 Armonk, NY 10504 USA
 frank@fordham.edu

Fritz Geiser

Centre for Behavioural and Physiological
 Ecology, Zoology
 University of New England
 Armidale, NSW 2351, Australia
 fgeiser@une.edu.au

Dennis Grahn

Department of Biological Sciences
Stanford University
Gilbert Hall
Stanford CA 94305 USA
dagrahn@stanford.edu

Gordon C. Grigg

University of Queensland
School of Life Sciences
Brisbane, Queensland 4072, Australia
ggrigg@zen.uq.edu.au

Michael B. Harris

Institute of Arctic Biology
311 Irving I Box 757000
University of Alaska Fairbanks
Fairbanks, AK 99775-7000 USA
ffmbh@uaf.edu

Gerhard Heldmaier

FB Biologie/Tierphysiologie
Philipps-Universität Marburg
D-35032 Marburg, Germany
heldmaier@staff.uni-marburg.de

Marcelo Hermes-Lima

Universidade de Brasília
Departamento de Biologia Celular
Campus Universitário, Asa Norte
70910-900 Brasília
DF Brazil
hermes@unb.br

Sara M. Hiebert

Swarthmore College
Department of Biology
500 College Ave
Swarthmore PA 19081 USA
shieber1@swarthmore.edu

Esa Hohtola

Department of Biology
University of Oulu
PO Box 3000
FIN-90014 Oulu FINLAND
esa.hohtola@oulu.fi

Murray M. Humphries

Department of Natural Resource Sciences
Macdonald Campus, McGill University
21,111 Lakeshore Road
Ste-Anne-de-Bellevue
Quebec H9X 3V9, Canada
humphries@nrs.mcgill.ca

Thomas L. Husted

Department of Surgery
University of Cincinnati College of Medicine,
Cincinnati, OH 45221, USA
thusted@pol.net

Donald C. Jackson

Brown University
Department of Molecular Pharmacology,
Physiology, and Biotechnology
Box G-B328, Brown University
Providence, RI 02912-G USA
donald_jackson@brown.edu

Martin Jastroch

Philipps University of Marburg
Department of Biology
Animal Physiology
Karl von Frisch Str. 8
D-35043 Marburg, Germany
jastroch@staff.uni-marburg.de

Barry G. Lovegrove

School of Botany and Zoology
University of KwaZuluNatal
Private Bag X01
Scottsville 3209, South Africa
lovegrove@nu.ac.za

André Malan

Université Louis Pasteur
Neurobiologie des Rythmes
12 rue de l'Université
F-67000 Strasbourg, France
malan@neurochem.u-strasbg.fr

Sandra Martin

School of Medicine
Cellular & Structural Biology
University of Colorado
Biomedical Research Building, Room 702
Campus Box B111, 4200 E. Ninth Ave.
Denver, CO 80262 USA
sandy.martin@uchsc.edu

Andrew E. McKechnie

Animal, Plant and Environmental Sciences
University of the Witwatersrand
Private Bag 3
Wits 2050, South Africa
mckechnie@gecko.biol.wits.ac.za

Eva Millesi

University of Vienna
Institute of Zoology
Althanstraße 14
1090 Vienna, Austria
eva.millesi@univie.ac.at

William K. Milsom

Department of Zoology
University of British Columbia
6270 University Blvd.
Vancouver, BC V6T 1Z4, Canada
milsom@zoology.ubc.ca

Nomakwezi Mzilikazi

School of Botany and Zoology
University of Natal-PMB
Private Bag X01
Scottsville, 3209
KZN-South Africa
992237121@nu.ac.za

Jan Nedergaard

Valeria Golozoubova
The Wenner-Gren Institute
The Arrhenius Laboratories F3
Stockholm University
SE-106 91 Stockholm, Sweden
jan@metabol.su.se

Stewart Nicol

Department of Anatomy & Physiology
University of Tasmania
Private Bag 24, Hobart
7001 Tasmania, Australia
s.c.nicol@utas.edu.au

Savvas C. Pavlides

Binghamton University (SUNY)
Department of Biological Sciences
Binghamton, NY 13902, USA
bg26598@binghamton.edu

Paul Pévet

Neurobiologie des Rythmes
UMR 7518 CNRS-Université L. Pasteur
12 rue de l'Université
F-67000 Strasbourg, France
pevet@neurochem.u-strasbg.fr

Irina Yu Popova

Institute of Theoretical and
Experimental Biophysics
Russian Academy of Sciences
Pushchino, 14 Leninskii pr.
Moscow 119991 Russia
i-yu-popova@yandex.ru

Donald R. Powers

Department of Biology & Chemistry
George Fox University
414 N. Meridian St.
Newberg, OR 97132 USA
dpowers@georgefox.edu

Norman F. Ruby

Department of Biological Sciences
Stanford University
371 Serra Mall
Stanford, CA 94305-5020 USA
ruby@stanford.edu

Steve Rudich

Department of Surgery
University of Cincinnati College of Medicine
231 Albert Sabin Way
P.O. Box 670558
Cincinnati, OH 45267-0558 USA
rudichs@ucmail.uc.edu

Donna G. Sieckmann

Resuscitative Medicine Department
Combat Casualty Care Directorate
Naval Medical Research Center, Code 032
503 Robert Grant Ave.
Silver Spring, MD 20910-7500 USA
sieckmannd@nmrc.navy.mil

Kenneth B. Storey

Institute of Biochemistry
Carleton University
1125 Colonel By Drive
Ottawa, ON K1S 5B6, Canada
kenneth_storey@carleton.ca

Glenn Tattersall

Department of Biological Sciences
Brock University
St. Catharines, ON L2S 3A1, Canada
gtatters@brocku.ca

Thomas E. Tomasi

Department of Biology
Southwest Missouri State University
901 S National Ave
Springfield, MO 65804, USA
tet962f@smsu.edu

Frank van Breukelen

Department of Biological Sciences
University of Nevada
4505 Maryland Parkway
Las Vegas, NV 89154, USA
frank.vanbreukelen@ccmail.nevada.edu

Eddy A. Van der Zee

University of Groningen
Departments of Molecular Neurobiology and
Animal Behavior
Kerklaan 30
9751 NN Haren Groningen, The Netherlands
e.a.van.der.zee@biol.rug.nl

Craig K.R. Willis

Centre for Behavioural and Physiological
Ecology
University of New England
Armidale NSW 2351, Australia
cwillis2@pobox.une.edu.au

Christopher P. Woods

Department of Biology
University of Regina
Regina, Saskatchewan S4S 0A2, Canada
chriswoods@velocity.net

Karl Erik Zachariassen

Laboratory for Ecophysiology and Toxicology
Department of Biology
Norwegian University of Science and
Technology
7491 Trondheim, Norway
karl.erik.zachariassen@chembio.ntnu.no

An Evolutionary Framework for Studies of Hibernation and Short-term Torpor

GORDON C. GRIGG

University of Queensland, School of Life Sciences, Brisbane, Australia 4072.

Abstract. Data from diverse studies endorse ideas that short term torpor and hibernation are expressions of ancient characters. In evolutionary terms, their basic mechanisms are probably plesiomorphic (= ancestral/primitive) and physiologically similar. This contrasts with the alternate view that they are apomorphic (= derived, specialized), arising independently in many taxa from homeothermic ancestry by numerous apparent convergences. This paper explores some of the implications of accepting the plesiomorphic interpretation. Hibernation is, of course, a complex phenomenon that has undergone variations and refinements in different mammalian lineages. The argument is not that hibernation in total is a plesiomorphic character, but that it is built upon fundamental processes that are. Taking this view provides a framework for research that emphasizes the value of comparative studies, particularly of reptiles and birds. Studies of reptiles, for example, might unravel the mystery about periodic arousals. A plesiomorphic framework also explains the most extreme examples of hibernation as derived specializations from ancestry in which heterothermy is more about energy management than escape from cold. It cautions against using low body temperature (T_b) alone to diagnose torpor, emphasizes the need to distinguish between constitutional eurythermy (plesiomorphic) and constitutional stenothermy (apomorphic), and leads to a parsimonious theory about the evolution of endothermy. The paper proposes that brown adipose tissue (BAT) is apomorphic within eutheria and highlights the conundrum posed by the occurrence of both nonshivering thermogenesis (NST) and rapid arousal from hibernation in noneutherian mammals that lack BAT and uncoupling protein 1 (UCP1). It

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endorses the likely existence of a different, ancient and widespread mechanism for regulatory NST in mammals.

Introduction

There are two conflicting views about the evolutionary history of hibernation. On the one hand is the view that hibernation is apomorphic (= derived, specialized), having arisen independently from homeothermic ancestry in separate groups by convergence (*sensu* Lyman and Blinks, 1959; Wang, 1989). On the other hand is growing support for the older idea that the physiological elements that support both hibernation and short term torpor are plesiomorphic (= primitive/ancestral). This paper argues in favor of the latter interpretation, suggesting that it offers a more coherent and more useful framework within which to understand the biology of heterothermy and its evolutionary history and significance within endotherms.

Most hibernation studies are framed within the context of assumptions that hibernation and short-term torpor are apomorphic. The recognition that they are probably plesiomorphic provides a quite different evolutionary context and one that should prove more useful for framing future studies. For example, when investigating the functional significance of periodic arousals from hibernation in mammals, it could be useful to look at reptiles, many of which show large daily swings in body temperature (T_b), are frequently cold throughout winter yet emerge and warm up from time to time on sunny days. Indeed, Rismiller and McKelvey (2000) described patterns of T_b in a varanid lizard in winter which appear strikingly similar to the spontaneous arousals that characterize hibernating mammals. Furthermore, if the fundamental elements of hibernation, daily torpor, and estivation are very ancient, this has implications for ideas about how endothermy itself may have evolved. Current models about the evolution of endothermy all focus on the evolution of homeothermic endothermy (Ruben, 1995, 1996; Hayes and Garland, 1995), and torpor and hibernation are dismissed as irrelevant, derived specializations (see Ruben, 1995). However, if short term torpor and hibernation are echoes of a time when early endotherms were facultative in their endothermy, information about the processes of entering and emerging from torpor could be very relevant to ideas about the evolution of endothermy, as discussed by Grigg and Beard (2000). A model for the evolution of endothermy that accommodates the probable plesiomorphy of short-term torpor and hibernation is proposed elsewhere (Grigg, Beard, and Augee, unpublished manuscript).

None of this, of course, implies that the spectacular capacities for hibernation seen in, for example, arctic ground squirrels, mountain pygmy possums and short-beaked echidnas are not functionally advanced. Rather, it implies that these capabilities are specializations from attributes that can be traced back to the first protoendotherms and, for some, the reptiles.

Evidence That Hibernation and Short-term Torpor are Plesiomorphic

The most compelling evidence that hibernation is plesiomorphic is that it occurs with essentially similar patterns in the three extant mammalian subclasses. This has been reviewed by Grigg and Beard (2000), who used it as a basis for discussing ideas about the evolution of endothermy. Such diversity of taxonomic occurrence, by parsimony, implies plesiomorphy (see below).

Hibernation was first described in eutherian mammals and for a long time was thought their exclusive preserve (Cossins and Barnes, 1996), although it was also described in a captive marsupial in the 1960s (see review by Geiser, 1985). However, field studies on short-beaked echidnas by Grigg et al. (1989, 1992) and Nicol and Andersen (1996) and then by Broome and Geiser (1995) on the marsupial mountain pygmy possum, *Burramys parvus*, revealed typical patterns of classical hibernation in both species. Hibernation is known in only one species of bird (French, 1993), but short-term torpor is common (see Geiser and Ruf, 1995; McKechnie and Lovegrove, 2002). I will focus mainly on mammals but, because both groups evolved from reptiles, even if endothermy arose independently in birds and mammals (Kumar and Hedges 1998) this does not preclude the physiology that supports torpor in birds sharing the same ancient origins.

Following the discoveries of eutherian-style hibernation in the monotremes and the marsupials, Augee and Gooden (1992) invoked parsimony to propose that the capacity for hibernation is plesiomorphic. They recognized that hibernation in these creatures is not primitive in any functional sense, being a "complex and highly adaptive phenomenon," and went on to conclude that the idea of separate derivations of a "complex phenomenon such as hibernation" in the Rodentia, Insectivora, Microchiroptera, the Monotremata, the Marsupialia (and perhaps Carnivora and Primates) "pushes the concept of convergence beyond its usefulness" (p. 175).

Most evolutionary biologists would agree that this alone provides compelling evidence. Moreover, Malan (1996) came to the same conclusion from an entirely

different line of reasoning. He noted similarities in the T_b patterns expressed by hibernators and torpidators to those seen seasonally in many reptiles and came quite independently to the conclusion that “we should now consider the phylogeny of hibernation, not as the repetitive and independent occurrence of a secondary adaptation in various phyla (*sic*), but simply as a recurring expression of primitive traits” (p. 2). Going further back, Cade (1964) analysed the distribution of torpor or hibernation (in five out of 14–15 distinct lineages) within the Rodentia and came to the conclusion that “torpidity is a manifestation of primitive organization in rodents” (p. 107). More recently, Lovegrove et al. (1999) interpreted the finding of daily torpor in elephant shrews (Macroscelidae) as support for a plesiomorphic origin of heterothermy.

If short-term torpor (commonly daily torpor) and hibernation were homologous, this would further endorse the ideas about plesiomorphy because, like hibernation, daily torpor also occurs in all three subclasses of mammals (as well as in birds) and across a wide diversity of taxa. There has been debate about whether short-term torpor and hibernation are quite different physiological phenomena, or are homologous, sharing a similar underlying mechanism expressed to different extents. In a comprehensive analysis, Geiser and Ruf (1995) compared attributes of torpidators and hibernators and concluded that there was a clear gap in the range of maximum torpor bout durations between daily heterotherms (1.5–22 h) and hibernators (96–1,080 hours), as well as differences in minimum oxygen consumption. However, Grigg et al. (1992) described both short-term torpor and long-term hibernation in the same animal, the short-beaked echidna, and more recently Kuchel (2003) found in several echidna-years of field observations that it was impossible to make distinctions between the two: one, two, and three day torpor bouts merged indistinguishably into weeks of classic hibernation with short periodic arousals. Metabolic measures also revealed no distinctions. Wilz and Heldmaier (2000) were unable to distinguish physiologically between daily torpor, hibernation, and estivation in the edible dormouse, *Glis glis*. They suggested “that all three forms of dormancy are based on the same physiological mechanism of thermal and metabolic regulation” (p. 511). Using the Geiser and Ruf (1995) criteria, Lovegrove et al. (2001) were unable to classify the patterns of heterothermy they found in two species of elephant shrew (*Elephantulus myurus* and *E. rozeti*) into either daily torpor or hibernation. They concluded that “these two physiological responses may not necessarily have separate evolutionary origins.” Overall, the weight of evidence favors their homology.

It seems reasonable to conclude that the physiological mechanisms that underlie both short-term torpor and hibernation are conservative and very old indeed. In addition, with ever-growing numbers of examples of heterothermy, particularly from studies of free-ranging mammals (and birds), the traditional statement in textbooks that endotherms characteristically have a high and stable body temperature should be accompanied by suitable qualification to accommodate the numerous and diverse exceptions.

Defining and Diagnosing Torpor

The proposed evolutionary framework will be helpful in diagnosing different types of torpor, and it emphasizes the need for a greater focus on behavior. Diagnosing short-term torpor by the extent of the temperature drop alone is quite limiting and, though expedient, may be unfortunate. McKechnie and Lovegrove (2002) have made a similar point for birds. Torpor is defined in the IUPS glossary as inactivity and reduced responsiveness (IUPS Thermal Commission, 2001), but in practice it is almost always diagnosed by the extent of the drop in T_b (e.g., Barclay et al., 2001), whether or not there is any reduced responsiveness. A temperature drop that leaves an animal still competent is obviously very different from one in which insensibility and immobility result. Short-beaked echidnas can be active and foraging apparently normally with a T_b of 20° C, i.e., 12° C below their modal temperature (Kuchel, 2003), and some other heterothermic endotherms show similar capacity. Many others become unresponsive and immobile with only a few degrees drop in temperature. McKechnie and Lovegrove (2002) have discussed the difficulty of distinguishing between torpor and “rest-phase hypothermia” in birds and emphasized the need to consider responsiveness to external stimuli. Grigg and Beard (2000) discussed the limitations posed by using terminology that describe pattern, not mechanism. Clearly, when mechanisms are better understood some new mechanism-based terminology may evolve (as ectothermy and endothermy replaced poikilothermy and homeothermy). In the meantime, it may be useful to recall the concept of “constitutional eurythermy” used by Eisentraut (1960) to refer to a capacity to be active over a wide range of T_b . Constitutional eurythermy is typical of reptiles and reflects underlying physiological capabilities. It is almost certainly the plesiomorphic condition. The constitutional stenothermy seen in many mammals (and birds), however, can be thought of as apomorphic, reflecting a loss of that capability. Monitoring hypothermia alone does not discriminate between eurytherms and stenotherms. Future physiological studies

of torpor probably need to pay more attention to the effect of lower T_b on the responsiveness of the subject.

An Evolutionary Framework for the Evolution of Short-term Torpors and Hibernation

If short-term torpor, hibernation, and constitutional eurythermy are plesiomorphic, this provides a unifying framework for thinking about the great diversities of heterothermy seen across the endotherms. The framework accommodates the growing number of examples of hibernation in mild climates (short-beaked echidnas) and even tropical climates (mouse lemurs, Dausmann et al., 2000) and where food is in good supply (see review by Grigg and Beard, 2000). It acknowledges that daily torpor is very common in mild and tropical climates (e.g., Ortmann et al., 1996). It sits well with the different extents of heterothermy seen in the three subclasses of mammals, correlating with the severity of the climate. For example, among the extant species of eutherian ground squirrels, a desert species, *Spermophilus tereticaudus*, relaxes its thermoregulatory limits and gains significant energy savings below the thermoneutral zone (Wooden and Walsberg, 2002); a temperate mountain species, *Cynomys ludovicianus*, shows facultative torpor (Lehmer et al., 2001); while an arctic species, *Spermophilus parryii*, shows an obligate, “classic” hibernation pattern complete with a long period of physiological preparation, metabolic defense against T_b falling below about -2.0°C and periodic arousals to normothermia (Boyer and Barnes, 1999; Barnes and Buck, 2000); presumably these capabilities became highly refined as arctic ground squirrels evolved from species in less demanding climates. A parallel pattern of torpor expression is seen in the marsupials. Within the Burramyidae, reviewed by Geiser (1985), some show only “energy management” daily torpors, some show longer torpors, while *Burramys parvus*, above the snow line, shows a “classic” pattern of hibernation (Broome and Geiser, 1995). Short-beaked echidnas (monotremes) show this whole range of patterns within a single species, depending on the climate, hibernating and avoiding harsh conditions for much of the winter above the snowline but showing variable, facultative patterns of “energy management” heterothermy elsewhere (Grigg et al., 1989, 1992; Grigg and Beard, 2000; Kuchel, 2003).

Implications for the Evolution of Endothermy

Despite their dismissal as irrelevant by Ruben (1995), capacities for short-term torpor and hibernation are probably quite central to ideas about the evolution

of endothermy. Assuming that torpor and hibernation are plesiomorphic, Grigg and Beard (2000) and Grigg et al. (unpublished manuscript) have proposed that the first protoendotherms were facultatively endothermic when appropriate for their activity, but retained their ectothermy (“constitutional eurythermy”) at other times. We argue that brooding endothermy in some otherwise ectothermic Boidae shows an incipient capacity for facultative endothermy in reptiles, and note that short-beaked echidnas provide a useful model of an (advanced) proto-endotherm, having the advantages of endothermy but minimizing its energetic costs by using ectothermy facultatively. In this model, observed patterns of T_b are a consequence of the underlying mechanisms and energy optimization, and homeothermy results when it is energetically desirable, rather than as the logical endpoint. Assuming that torpor in mammals is an echo of their reptilian ancestry implies that studies of short-term torpor and hibernation and the mechanisms that support them may be very relevant to gaining an understanding of the details of how endothermy evolved.

Implications for Studies of BAT

Searches for UCP1 using molecular techniques have confirmed what Hayward and Lisson (1991) concluded from their survey of the mammals using microscopy: that brown adipose tissue (BAT) is lacking in marsupials and monotremes (and birds) and is unique to Eutheria. It probably evolved early in the eutherian radiation, and it can be regarded as apomorphic. It is not diagnostic of mammals, despite the implication made by Cannon and Nedergard (2004).

Intriguingly, although the capacity for both NST and the periodic arousals characteristic of the “classic” hibernators are attributed in textbooks to BAT and its uncoupling protein UCP1, BAT is apparently not needed for arousal from hibernation in either monotremes or marsupials. The rapid arousals from hibernation in *Burramys* and short-beaked echidnas are markedly similar to those seen in eutherians. Furthermore, Rose et al. (1999) and Kabat et al (2003a, b) have clearly demonstrated that norepinephrine-stimulated NST occurs in cold-acclimated Tasmanian Bettongs, *Bettongia gaimardi*, and Tasmanian devils, *Sarcophilus harrisii*, both of which are homeothermic endotherms that lack both BAT and UCP1.

This conundrum requires explanation. Grigg and Beard (2000) pointed out that although “BAT may be an important accessory site for thermogenesis, so many endotherms lack BAT that it can be only one part of a larger story,” The larger story seems to be emerging now, in birds at least, with the finding that

birds show facultative (regulatory) NST originating probably from the sarcoplasmic reticulum (Bicudo et al., 2001, 2002). The skeletal muscle of mammals, too, is a likely source of regulatory NST. Ye et al. (1995, 1996), aware of the lack of BAT in marsupials, explored other possible sources of NST in *B. gaimardi* and found that catecholamines modified skeletal muscle oxygen consumption and lactate and glycerol production. They concluded that the skeletal muscle vascular bed made a significant contribution to whole-body thermogenesis.

It seems likely that a widespread, ancient, and controllable mechanism of regulatory NST will be found in mammals as well. Whether it will be the same in mammals as in birds remains unknown. If it were the same, that would be very provocative for current ideas about the separate origins of endothermy in these two groups.

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Was Adaptive Hypothermia a Prerequisite for the Colonization of Madagascar By Mammals?

BARRY G. LOVEGROVE

School of Botany and Zoology, University of KwaZuluNatal, Scottsville, South Africa

Abstract. It has often been suggested that adaptive hypothermia must have been a prerequisite for terrestrial mammals (tenrecs, rodents, primates, and carnivores) to make the crossing from Africa to Madagascar by rafting. The potential importance of torpor during colonization events was investigated by seeking evidence of torpor in the mainland relatives of the colonizing species and in the extant Madagascan lineages. The Tenrecidae are the only Madagascan mammals for which a convincing argument for the importance of “rafting torpor” can be made. Reconstructions of ancestral body sizes of the colonizing rodent and primate suggest that they may have been larger than the body size of mammals that typically employ torpor.

Introduction

Several authors have suggested that the successful colonization of Madagascar by “sweepstakes” over-water dispersal (e.g., by rafting) was made possible by the employment of daily torpor or hibernation (Kappeler, 2000; Yoder et al., 2003). Such claims require physiological verification in terms of our understanding of the evolution of torpor and the role that adaptive hypothermia may have played in biogeography and species dispersal.

At the time that the Indo-Madagascan continent separated from Africa 165 mya, the eutherian mammals had yet to evolve (Murphy et al., 2001; Eizirik et al., 2001) (Fig. 1). Nevertheless, Madagascar hosts four eutherian lineages, the Tenrecidae, Primates (lemurs), Rodentia (Nesomyinae), and Carnivora (Garbutt, 1999). All Malagasy eutherian orders are monophyletic, suggesting that

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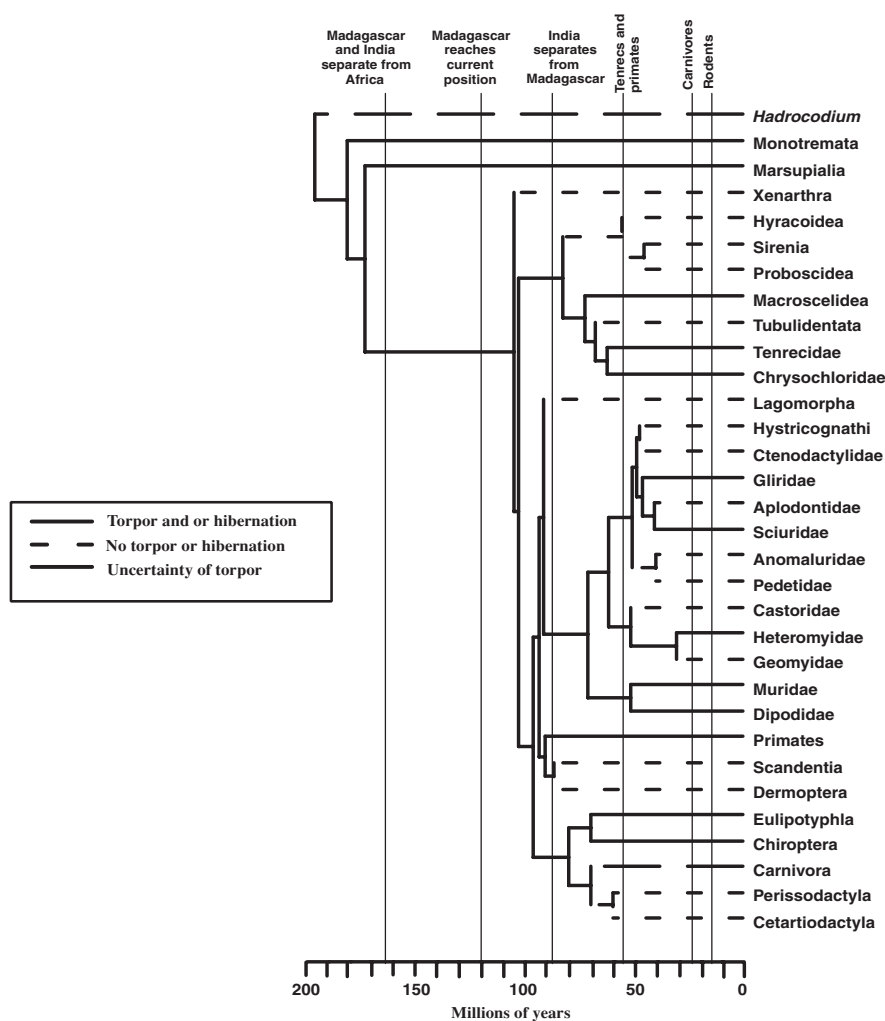


Fig. 1. The phylogeny of mammalian orders and families shows where pleisiomorphic adaptive hypothermia has been either lost or retained. The vertical dotted lines indicate important geological events and the earliest estimated colonization times of the four lineages of Malagasy mammals. Topology (Murphy *et al.*, 2001; Eizirik *et al.*, 2001), divergence dates; orders (Eizirik *et al.*, 2001), rodents (Michaux and Catzeflis 2000; Adkins *et al.*, 2001; Huchon and Douzery 2001; Michaux *et al.*, 2001). Colonization estimates; Tenrecidae (Douady *et al.*, 2002), Rodentia (Michaux and Catzeflis 2000), Primates (Yoder *et al.*, 2003), and Carnivora (Yoder *et al.*, 2003).

Madagascar was colonized by a single ancestor of each order once only (Jansa et al., 1999; Douady et al., 2002; Yoder et al., 2003).

The tenrecs and the primates were first to colonize Madagascar from Africa at roughly the same time, ca. 55 mya (Yoder et al., 1996; Douady et al., 2002). The most likely origin of the nesomyine rodents is from the ancient cricetine African lineage (Michaux and Catzeflis, 2000; Michaux et al., 2001), although other origins have been proposed (Jansa et al., 1999). Based upon a molecular clock estimate of the divergence of the Nesomyinae from their closest African relatives, the Mystromyinae, the earliest possible timing of the colonization event by rodents is 14–15 mya (Michaux et al., 2001). The carnivores arrived in Madagascar 24–18 mya (Yoder et al., 2003).

This chapter traces the inheritance of adaptive hypothermia throughout the mammalian phylogeny in order to evaluate it as a prerequisite for the successful colonization of Madagascar. The closest living mainland relatives of the colonizers are identified to verify whether or not they retain pleisiomorphic daily torpor and/or hibernation (Geiser, 1998; Lovegrove et al., 1999b).

Discussion

The Tenrecidae

The tenrecs are most closely related to the Chrysochloridae (golden moles), Macroscelidae (elephant shrews), and perhaps the Tubulidentata (aardvark's position within Afrotheria remains uncertain; see Murphy et al., 2001; Eizirik et al., 2001) (Fig. 1). These are members of the Afrotheria, the endemic African clade that includes the elephants, manatees, and hyraxes and which, together with the South American Xenarthra, are the basal eutherians (Springer et al., 1997; Murphy et al., 2001). Elephant shrews and golden moles are known to employ daily torpor (Fielden et al., 1990; Lovegrove et al., 1999b; Lovegrove et al., 2001; Mzilikazi and Lovegrove, 2002; Mzilikazi and Lovegrove, 2004). Within the Afrotheria, they are the smallest in size—all other Afrotherians are larger than 1 kg and have lost the capacity for daily torpor (Fig. 1).

Conclusions about whether tenrecs use torpor can be drawn from thermoregulatory studies (Stephenson and Racey, 1993a, 1993b; Stephenson et al., 1994; Stephenson, 1994). For example, *Geogale aurita* displayed patterns of metabolic and T_b reduction below thermoneutrality (Fig. 2) identical to those of typical daily heterotherms (Geiser and Ruf, 1995). Body temperature was reduced to ca. 16° C, which is about the average for most daily heterotherms (Geiser and Ruf, 1995). Tenrecs also seem to display remarkable ectothermic-like responses to ambient

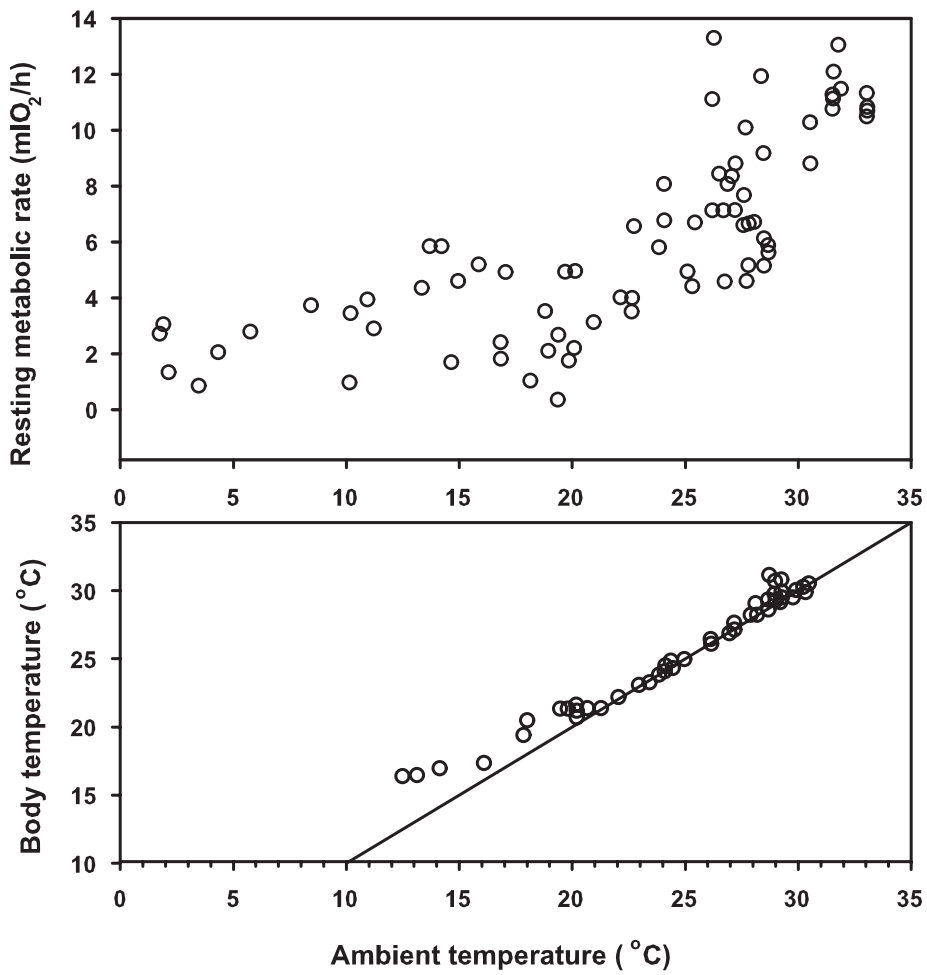


Fig. 2. (Top) Resting metabolic rate and (bottom) body temperature as a function of ambient temperature in the large-eared tenrec, *Geogale aurita*. Data digitized and redrawn from Stephenson and Racey (1993a).

temperature during which, irrespective of the T_a , T_b is maintained ca. 1°C higher than the ambient temperature (Nicoll, 1986). The T_b of an individual *Tenrec caudatus* implanted with a telemeter and maintained in a refrigerator in which the temperature was varied throughout the day from $14\text{--}28^\circ\text{C}$ was closely synchronized with ambient heating and cooling curves (Fig. 3), a pattern seen during passive heating during arousal in elephant shrews (Mzilikazi and Lovegrove, 2002), some small marsupials (Lovegrove et al., 1999a; Geiser and Drury, 2003), and Malagasy dwarf lemurs (Ortmann et al., 1996; Schmid, 2001).

Thus since both the closest mainland relatives of the tenrecs and the Madagascan tenrecs themselves have retained the use of torpor, we cannot exclude the role of adaptive hypothermia during colonization in this group.

Primates

Several species of Malagasy cheirogalid dwarf and mouse lemurs (*Microcebus* and *Cheirogaleus* spp.) use torpor (Schmid, 1996; Ortmann et al., 1996; Schmid,

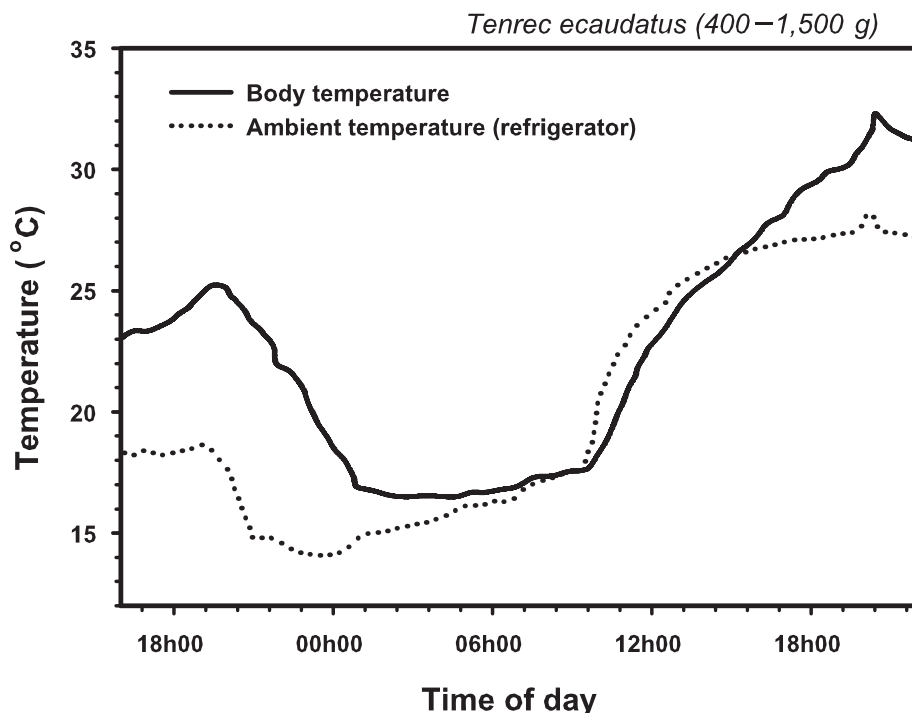


Fig. 3. The telemetered intraperitoneal body temperature of the tenrec, *Tenrec ecaudatus*, held in a refrigerator. Data digitized and redrawn from Nicoll (1986).

2000; Schmid et al., 2000; Schmid, 2001). Kappeler (2000) has argued that their capacity for torpor underlies successful colonization because “entire groups of animals survived the weeks or even months of such a journey without food or water sleeping in a hollow tree while rafting across the sea.” Apart from verifying pleisiomorphic torpor in the closest mainland relatives, an additional question relative to this argument is what the body size of the ancestral colonizer was likely to have been?

The Malagasy primates are monophyletic, but the Cheirogalidae are not basal (Yoder and Irwin, 1999; Yoder et al., 2003). The basal genus is *Daubentonia*, the aye-aye (2,800 g). Moreover, the predicted body size of a related species, *Daubentonia robusta* that went extinct ca. 1,500 years ago following the colonization of Madagascar by man, was about 13.5 kg. Thus it is not obvious whether the current small size of the cheirogalids is a consequence of rapid character displacement and radiation following colonization, or whether a small size was inherited from the common ancestor of *Daubentonia* and the cheirogalids.

We have reconstructed the body size of the colonizing ancestral lemur using a local and global parsimony linear contrasts approach (see Garland et al., 1999) calculated from a complete phylogeny of the primates, including the various genera of large lemurs that also were driven to extinction by man within the past 1,500 years (e.g., *Palaeopropithecus* and *Hadropithecus*) (Masters and Lovegrove, unpubl.). The mean body masses of 71 primate genera were calculated (data from Godfrey et al., 1995; Rowe, 1996; Godfrey et al., 1997) and entered as \log_{10} -transformed tip values into a primate phylogeny constructed from molecular and morphological data (Adkins and Honeycutt, 1994; Oates et al., 1994; Rumpler et al., 1994; Jacobs et al., 1995; Lockwood, 1995; Disotell, 1996; Porter et al., 1997; Goodman et al., 1998; Collins and Dubach, 2000; DelPero et al., 2000; DelPero et al., 2001; Pastorini et al., 2001; Collins and Dubach, 2001; Montagnon et al., 2001; Page and Goodman, 2001; Godfrey and Jungers, 2002, 2003; Masters et al., in press). After rerooting the tree at the node subtending the Malagasy lemurs, the mean size of the ancestral lemur was estimated at 3,230 g, with lower and upper 95% confidence intervals of 792 g and 13,161 g, respectively. The primate that colonized Madagascar was therefore not necessarily a small mammal. Its reconstructed mass did not fall within the range of body masses of typical daily heterotherms (Geiser and Ruf, 1995); the lower body mass estimate does not overlap with the upper limit of the largest living cheirogalid (*Cheirogaleus major*, 500 g) (Garbutt, 1999).

The Malagasy lemurs are most closely related to the Galagidae (bush babies) of Africa, and the Loridae (lorises) of Africa and Asia (Martin, 2003). There are very few data on torpor in these groups. Studies of body temperatures of free-ranging lesser bush babies *Galago moholi* (210 g) failed to detect torpor (Mzilikazi et al., this volume), which does not necessarily obviate its existence in other smaller bush babies such as *G. demidovi* (97 g). Apart from bush babies, an observation of a single slender loris (*Loris tardigradus*) that attained a body temperature of 14.8° C when placed in a refrigerator (Müller et al., 1985) is the only evidence to date of possible adaptive hypothermia in strepsirhines. Thus adaptive hypothermia may have been retained in at least one strepsirhine family, the Loridae, and if confirmed, would suggest that the ancestral strepsirhine retained the trait. It is theoretically feasible, therefore, that the ancestral lemur inherited the trait as well.

If the ancestral lemur, by virtue of its presumed large body size, had lost the capacity for adaptive hypothermia, then torpor evolved independently a second time in the Cheirogalidae. This possibility would represent the only known case of independent evolution of torpor in the mammals (Fig. 1), which is considered highly unlikely given the suite of genes involved in the physiological response (Malan, 1996; Geiser, 1998). Thus the resolution of the argument for rafting torpor in primates hinges on our knowledge of torpor in the closest mainland relatives of the cheirogalids, the Loridae and the Galagidae. The current paucity of evidence of torpor in these two primate families thus clearly warrants research attention.

Rodents

There are, to my knowledge, no data on the energetics of any of 20 species of Malagasy nesomyine rodents. Thus we do not know whether these rodents are capable of torpor or hibernation. The species range in size from the mountain mouse (*Monticolomys koopmani*, 25 g) to the giant jumping rat (*Hypogeomys antimena*, 1.3 kg) (Garbutt, 1999). Thus body size, which often tends towards gigantism in island small mammals (Lomolino, 1985), does not preclude the use of torpor in several of the smaller Malagasy rodents. But do their mainland closest relatives display torpor?

The Nesomyinae form a sister family within a monophyletic African clade that includes the Mystromyinae, Cricetomyinae, and Dendromurinae (Michaux et al., 2001). Torpor has been recorded in the Cricetomyinae (*Saccostomus*) (Lovegrove and Raman, 1998) and the Dendromurinae (*Steatomys*) (Richardson

1990), but not in the single species within the *Mystromyinae*, *Mystromys albigaudatus*, although the prospect in the latter cannot be ruled out. Thus there is a reasonable phylogenetic argument for the inheritance of adaptive hypothermia in Malagasy rodents.

However, again consideration of the possible body size of the colonizing rodent warrants caution when speculating about whether the ancestor relied on torpor to reach Madagascar. The reconstructed body size of the colonizing Malagasy rodent, using the same approach described earlier for primates, was 103.9 g, with lower and upper 95% confidence intervals of 35.5 g and 302.7 g, respectively. This phylogeny employed the nesomyine topology (Jansa et al., 1999) placed within the topology of other African rodent families that employed a reliable outgroup (Michaux et al., 2001). The mean estimate suggests that the ancestral colonizer was a fairly large-sized rodent relative to the sizes of contemporary rodents, thus questioning whether or not the ancestor used torpor. Interestingly, this analysis also revealed that the only African mainland rodent that displays a significant trend towards gigantism is the African giant rat, *Cricetomys gambianus* (2.8 kg) which, as a sister species to *Saccostomus* in the *Cricetomyinae*, may have shared a common large-sized ancestor with the *Nesomyinae*.

Thus for rodents, no definitive conclusions can be drawn about the role of adaptive hypothermia in the colonization process until data on the thermal biology of the Malagasy *Nesomyinae* are made available. Moreover, it will also be necessary to evaluate the unusual gigantism in the mainland *Cricetomys* to establish whether the selection pressures were in any way involved with those operative on offshore islands.

Carnivora

Apart from various small carnivores introduced into Madagascar by man (Garbutt, 1999), there are eight native Malagasy carnivores derived from a mongoose (*Herpestidae*) ancestor (Yoder et al., 2003). Like their mainland sister taxa, none of them are known to employ daily torpor or hibernation. Moreover, the two other closely related mainland families (*Felidae* and *Hyaenidae*) also do not use torpor (Fig. 4). Thus the physiological capacity for adaptive hypothermia was lost in the felid-hyaena-herpestid-viverid lineage ca. 40 mya ago, but was retained in the other monophyletic carnivore clade that includes the *Canidae*, *Ursidae*, *Mustelidae*, *Procyonidae*, *Phocidae*, and *Otoariidae* (Fig. 4). Of course, the latter is true only if we regard the shallow torpor displayed by

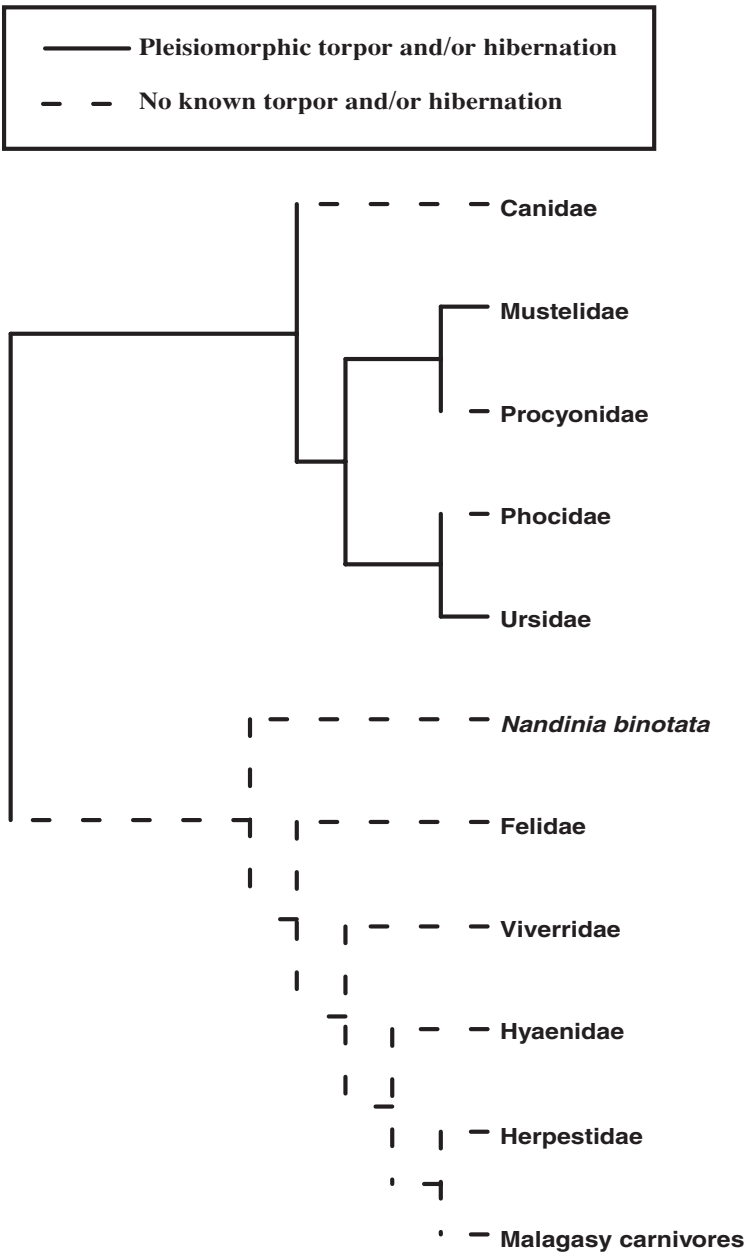


Fig. 4. The phylogeny of carnivore families, showing the loss of adaptive hypothermia throughout the monophyletic felid-hyaena-civet-mongoose clade. Topology Bininda-Emonds et al., 1999; Yoder et al., 2003.

bears and *Meles meles* (Geiser and Ruf, 1995) as being a synonymous physiological response to those of all other mammals that display adaptive hypothermia.

Thus the ancestral Malagasy carnivore was able to colonize the island without the aid of adaptive hypothermia. This is an important observation because it suggests that carnivores, which typically display high basal metabolic rates (Hayssen and Lacy, 1985; Lovegrove, 1996; McNab, 2000a, 2000b), were not constrained by energetic criteria during the colonization process. Was waif dispersal by rafting therefore unlikely to have been the mechanism whereby carnivores colonized Madagascar?

Concluding Remarks

Given the paucity of data on adaptive hypothermia in all Malagasy mammals, it would be premature to argue that adaptive hypothermia was a prerequisite for the colonization of Madagascar by rafting. At present, the tenrecs and the lemurs are the only Malagasy mammals in which torpor seems to have been inherited from a common ancestor. However, the origin of torpor in the Malagasy primates is intriguing and is undoubtedly worth pursuing to confirm whether or not torpor may have evolved more than once in mammals.

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No Evidence for Torpor in a Small African Mainland Primate: The Lesser Bushbaby, *Galago moholi*

NOMAKWEZI MZILIKAZI,¹ BARRY G. LOVEGROVE,¹ AND JUDITH C. MASTERS²

¹ School of Botany and Zoology, University of KwaZulu-Natal, Private Bag X01, Scottsville, South Africa

² Natal Museum, Private Bag 9070, Pietermaritzburg, 3200, South Africa

Abstract. The investigation of heterothermy in the strepsirrhine primates has focused largely on the Malagasy cheirogaleids. No data exist on the thermal biology of free-ranging African galagids. We measured body temperatures of 11 free-ranging *Galago moholi*, captured between February 2002 and September 2003, for three consecutive months for each animal. We expected lesser bushbabies to employ daily heterothermy during winter. However, we did not record any incidents of heterothermy throughout the study period. Why does *G. moholi* not employ heterothermy? We ponder several alternatives, including phylogenetic placement and ecological factors such as predation pressure, feeding habits, and breeding pattern. We suggest that the breeding pattern observed in *G. moholi* prevents torpor use while increasing fecundity, which would be adaptive if the animals are subjected to high predation risks.

Introduction

The investigation of heterothermy in the strepsirrhine primates has largely focused on the Malagasy cheirogaleids (Schmid, 1996; Ortmann et al., 1996; Schmid and Kappeler, 1998; Aujard et al., 1998; Schmid, 2001). Only one record of heterothermy has been reported in the Southeast Asian Lorisidae, the slender loris, *Loris tardigradus* (Müller et al., 1985). There has been little investigation of heterothermy in other strepsirrhines. The expression of heterothermy by Malagasy primates is important for approaching the question of whether

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

torpor is a plesiomorphic character that may have been inherited from an African mainland ancestor. However, the information on the thermal biology of the African Galagidae is scanty.

The southern African lesser bushbaby, *Galago moholi* (ca. 200 g) is a nocturnal savanna woodland species and in South Africa is usually associated with *Acacia* and mopane bush veld (Skinner and Smithers, 1990). Lesser bushbabies subsist mainly on gum that exudes from certain *Acacia* species, a diet frequently augmented by insects (Skinner and Smithers, 1990; Caton et al., 2000). Their feeding habits (Skinner and Smithers, 1990), phylogenetic placement (DelPero et al., 2000), body size (Geiser, 1998), zoogeographical location (Lovegrove, 1996, 2003), and behavior (Bearder and Martin, 1980) render them excellent candidate employers of heterothermy. However, except for a single study using captive-bred animals (Knox and Wright, 1989), the thermal biology of the lesser bushbabies has not been investigated. It is known that thermoregulation may differ markedly between captive and free-ranging animals (Geiser et al., 2000). The aim of this study was therefore to investigate the use of heterothermy (if any) by free-ranging southern lesser galagos, *Galago moholi*.

Study Site

The study site was located in the Nylsvlei Nature Reserve (Permit number 07877; Limpopo Province Department of Agriculture and Environment), Limpopo Province, South Africa (24° 29' S; 28° 42' E) and is comprised of semiarid, mixed bushveld habitat. The hot wet season lasts from November to March, and the cool, dry season from April to October. The reserve receives a mean annual precipitation of 630 mm. The annual mean temperature is 19° C and ranges between -3.2 and 23° C. Minimum temperatures below 0° C may be recorded between May and August (Scholes and Walker 1993).

Materials and Methods

The study was conducted between February 2002 and September 2003. All the procedures in this study complied with the Principles of Animal Care publication no. 86-23, revised 1986 (National Institute of Health) and the Code of Ethics for Animal Experimentation manual adopted by the University of KwaZulu-Natal. The animals were captured using walk-in live traps mounted on *Acacia* trees, which were baited during the night with a mixture of peanut butter, bananas, and honey and checked just before sunrise. We measured body temperature of 11 free-ranging *Galago moholi*, captured at different times

throughout the study period: February–May 2002 (two males), June–August 2002 (one female), October–December 2002 (four males), and June–August 2003 (one female; three males). Precalibrated Thermocron iButtons (temperature dataloggers; Dallas Semiconductor; accuracy 1°C from -20° to 70°C) were surgically implanted into the peritoneal cavities of the bushabies under inhalation anaesthesia (Isoflurane in oxygen; induction and maintenance, 2% flow rate, ca. 0.5 l min^{-1}). The surgical procedures did not have any adverse effects on the animals as they recovered within 60 minutes of surgery. They were released at their exact capture locations < 24 hours after initial capture. We programmed iButtons to measure T_b once every hour, resulting in a total of 2,040 data points per animal over a period of 85 consecutive days. To ensure that the animals had fully recovered and that surgery did not affect body T_b , we programmed the iButtons to start recording at least 15 days after surgery.

Since tree holes are used for resting, we placed an iButton in a shallow tree hole to measure (T_a) in a typical resting site of the lesser bushabies.

To show the relatively recent radiation of the galagos as well as the genera in which heterothermy is observed in the strepsirrhines (see Discussion), we constructed a composite phylogeny of extant strepsirrhines, comprising those nodes that received substantial support in the following phylogenetic analyses: Adkins and Honeycutt (1994), DelPero et al. (2000, 2001), Masters et al. (in press), Pastorini et al. (2001), Porter et al. (1997), and Rumpler et al. (1994).

Results

The mean body mass at initial capture was $182.5 \pm 35.6\text{ g}$ and the mean body mass at recapture was $193.4 \pm 24.8\text{ g}$ ($n = 11$). Year-round measurement of T_b s revealed no evidence of torpor in free-ranging *G. moholi*. Throughout the study period T_b did not decrease below 33°C in any of the animals (Fig. 1). The minimum T_b s during the day ranged from 33.1 – 38.2°C and the maximum T_b s during the day ranged from 35.9 – 39.7°C . On average, the daily minimum T_b , average T_b and maximum T_b were higher than the night T_b values (Fig 1). The mean T_b ranged from 34.8 – 38.6°C and 36.2 – 39.1°C , during the day and night respectively. The day maximum T_b ranged from 35.9 – 39.7°C and the night T_b maximum from 36.2 – 39.5°C (Fig. 1).

We expected the animals to exhibit torpor during the coldest part of the year (June–August). However, typical T_b plots (e.g., adult male; Fig. 2) of all the animals during mid-winter showed no evidence of torpor. The T_b patterns observed were consistent with the nocturnal activity pattern that we observed

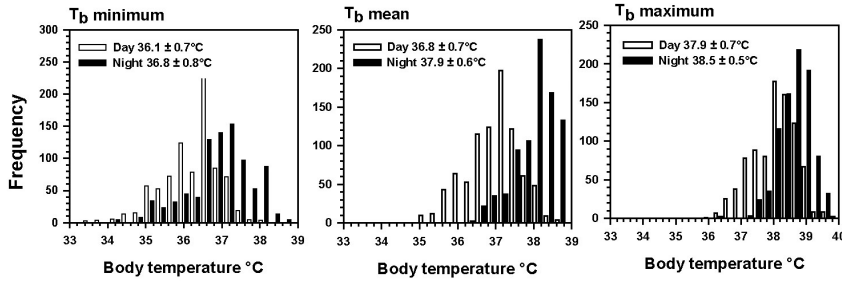


Fig. 1. Frequency distributions of minimum, average, and maximum T_b in free-ranging *G. moholi* between February 2002–August 2003.

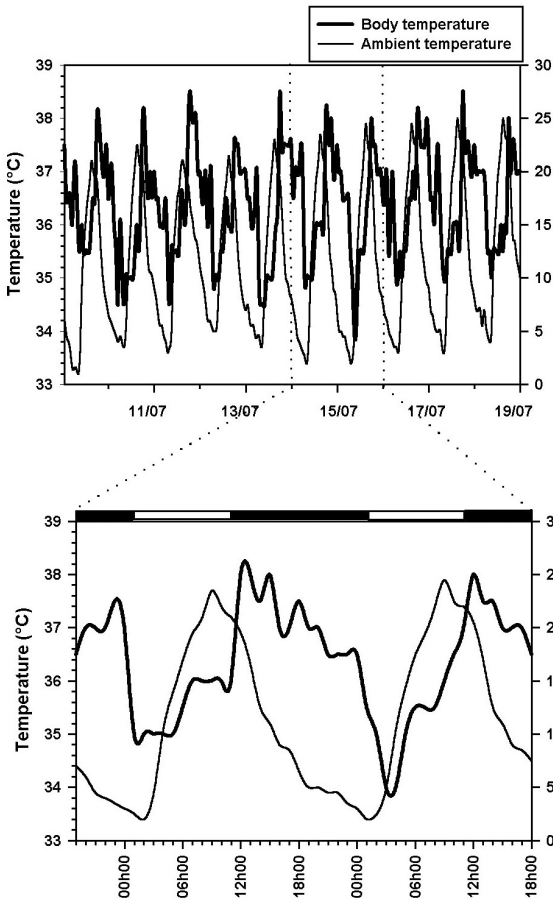


Fig. 2. Daily patterns of body temperature (T_b) measured in the peritoneal cavity of a free-ranging male *G. moholi* over a 10-day period in July 2003. The ambient temperature (T_a) was measured in a nearby tree hole. The dark bars on the top of the expanded lower two-day figure indicate the nighttime.

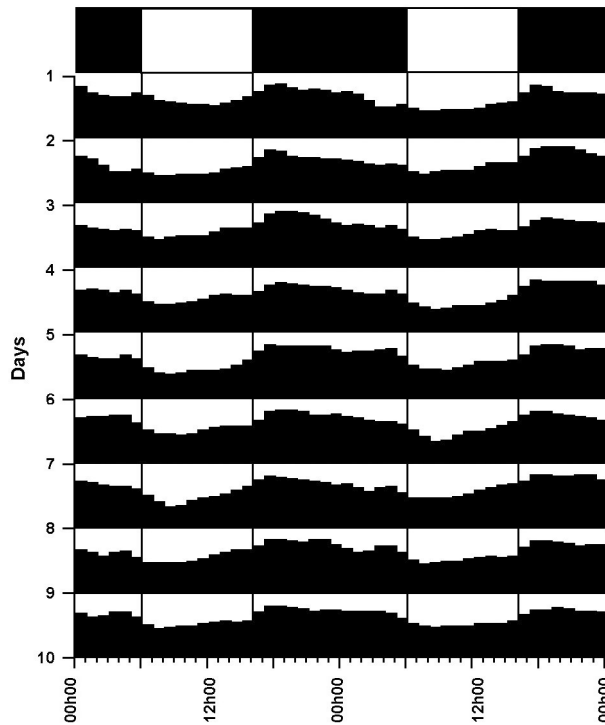


Fig. 3. Circadian double plots of body temperature of an individual free-ranging male *G. moholi*. The T_b scale ranges from 32–39° C in each plot of each day. The dark bars on top indicate the nighttime and the clear bars indicate the daytime.

(Fig. 3). The minimum body temperatures were attained in the two-hour period around sunrise, whereas the maximum T_b s were attained in the two-hour period around sunset (Figs. 2, 3).

Discussion

This study represents the first account of body temperatures in a free-ranging African strepsirrhine primate. We did not record any incidence of torpor in any of the 11 animals recorded during different seasons over the period of the study (February 2002–September 2003). We expected lesser bushabies to employ daily heterothermy during winter based on their body size, food habits, zoogeographical location, and behavior. A question of interest is why does *G. moholi* not employ heterothermy? We consider several alternatives.

Phylogenetic Placement

The observation that heterothermy was not found in *G. moholi* does not preclude the use of heterothermy by other members of the Galagidae. For example, the smallest members of the family may well use torpor. However, it does raise some interesting questions regarding the evolution of torpor in primates. The only primates for which detailed studies of heterothermy use are available are the Malagasy cheirogaleids (Ortmann et al., 1996; Schmid and Kappeler, 1998; Aujard et al., 1998; Schmid, 1998; Schmid, 2001). The cheirogaleids are the least specialized of the lemuroid families and have been interpreted by some to be closest to the ancestral strepsirrhine condition (Charles-Dominique and Martin, 1970). If torpor is a plesiomorphic character in mammals (Malan 1996), it is not unexpected that the cheirogaleids—all ≤ 600 g, nocturnal, and omnivorous (Garbutt, 1999)—should retain the ancestral condition of adaptive heterothermy by virtue of small body size (Geiser, 1998), phylogenetic placement and zoogeographical considerations (Lovegrove, 1996; Lovegrove, 2003). The lesser galago radiation appears to be fairly recent (Fig. 4; Masters, 1998; DelPero et al., 2000). Because torpor in mammals occurs in the phylogenetically older groups (Geiser, 1998), phylogenetic placement of the lesser galago may explain the lack of torpor.

Predation Pressure

G. moholi share morphological, behavioral, and ecological characteristics with the cheirogaleids. One possible explanation for absence of torpor in the lesser galago compared with the cheirogaleids may lie in the different predation pressures on the mainland and on Madagascar. The heterothermic state decreases responsiveness to external stimuli and may render animals vulnerable to predation. The cheirogaleids are prey for a number of mammalian, reptilian, and avian species, including the ring-tailed and narrow-striped mongoose, the long-eared Madagascar owl, the Madagascar barn owl, the Madagascar harrier-hawk, as well as tree and ground boas (Garbutt, 1999). We assume that the greatest risk of predation would be during the night when the animals are torpid (Schmid, 1996). We exclude reptiles and birds of prey from this argument because the reptiles presumably decrease activity during winter nights (when animals are torpid and most vulnerable) and the activity times for the predators (owls) and the prey do not overlap when torpor is used. We therefore only consider the mammalian Carnivora.

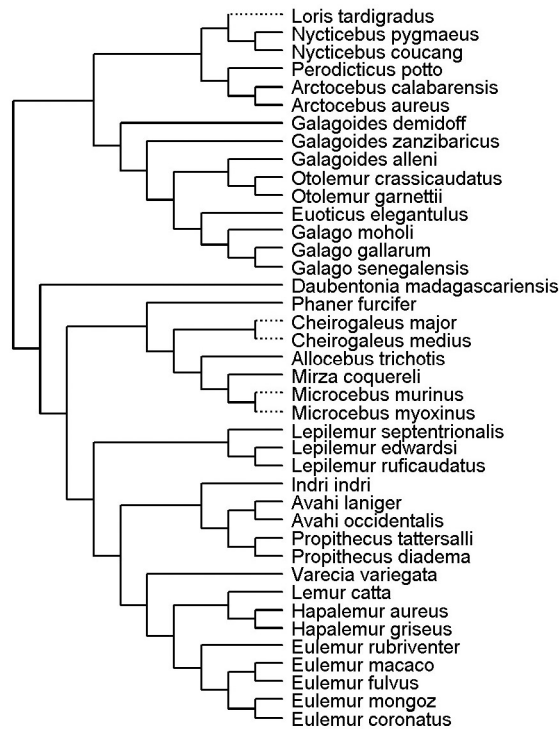


Fig. 4. A composite phylogeny of the strepsirrhine primates showing the relatively recent radiation of the lesser galagos (genus *Galago*). The horizontal dotted lines show species in which heterothermy has been observed. Note the relative paucity of heterothermy in strepsirrhines other than the cheirogaleids.

The primates are estimated to have colonized Madagascar ca. 65 mya, about 40 million years before the arrival of the first carnivores (Yoder et al., 2003; Lovegrove, this volume). Thus, the expression of heterothermy may have been retained because of the lack of early predation by mammals. However, body armour did evolve in the tenrecs, suggesting early predation pressure from non-mammalian carnivores such as reptiles and birds.

There is a longer association between the mainland carnivores and mainland strepsirrhines, and this association may have obviated the use of heterothermy by *G. moholi*. Tree holes may offer relative safety during torpor. However, *G. moholi* do not use tree holes exclusively and in our study site typically slept in open-top nests that were about 4–6 m above ground in thorny trees (*Acacia*

nilotica and *A. tortilis*) as well as in forked tree branches. At our study site, lesser bushbabies are preyed upon by a number of species, including mongooses, genets, owls, eagles, and snakes. We have occasionally found bushbabies attacked and killed by predators inside the traps. In predator-rich environments, the decreased responsiveness during heterothermy would subject small daily heterotherms to high predation pressures. Thus, high risks of predation may also act to select against use of heterothermy in this species.

Food Habits

Geiser (1998) argued that the use of heterothermy is most likely to be expressed by those taxa whose members are small and experience seasonal or aseasonal shortages in their food supply. The mean body mass for daily heterotherms is 253 ± 166 g, with the majority of daily heterotherms being < 100 g (Geiser and Ruf, 1995). The lesser bushbaby is within this size range and mainly on gum, and arthropods (Bearder and Martin, 1980). In our study site, insect availability decreases during winter but gum is available throughout the year owing to activities of wood-boring beetles and moths. The total activity budget spent gum foraging increases from summer to winter in lesser galagos (Bearder and Martin, 1980; Harcourt, 1986). Increased gum foraging coupled with curtailed overall activity has been explained as a mechanism to enhance survival in *G. moholi* during the winter months (Bearder and Martin, 1980). These authors suggested that it was doubtful whether survival would be possible without access to gum. However, they also reported on incidents during a particularly harsh winter where lesser bushbabies experienced considerable weight loss, high mortality, and loss of portions of their tails as a result of frostbite. Thus, during severe winters, the animals may not obtain adequate caloric intake from gum to meet their thermoregulatory requirements. It therefore seems highly likely that because of the apparent lack of a physiological capacity for heterothermy, *G. moholi* may suffer high mortalities during particularly harsh winters.

Breeding Pattern

The lack of heterothermic capacity may be associated with the breeding pattern observed in *G. moholi*. They breed during the winter months with mating observed during May. The gestation period is ca. four months, meaning that the females are pregnant throughout winter (Pullen et al., 2000). In addition, the females display postpartum oestrus, with a subsidiary mating season in October and subsequent births in February (Pullen et al., 2000). All the adult

male animals we captured throughout the study period had scrotal testes, implying a lack of testicular regression even during the cold winter months. In the lesser bushbabies, therefore, the females are not reproductively active for a short period only, between February and May, and the males seem to maintain a state of physiological readiness for reproduction throughout the year. The main difference between *G. moholi* and the cheirogaleids is that *G. moholi* mates at the beginning of winter (May) whereas in the cheirogaleids, mating is a summer event. Furthermore, the lesser bushbaby gestation period, ca. four months, is longer than in the cheirogaleids (ca. two months; Garbutt, 1999). Low ambient temperatures, particularly in our study site where midwinter temperatures may sometimes decrease below freezing, and decreased invertebrate availability in winter are conditions that we expected to trigger the use of torpor in lesser bushbabies. However, with the exception of marsupials, reproductive activity with its concomitant high levels of reproductive hormones and use of heterothermy are mutually exclusive (Goldman et al., 1986; Barnes et al., 1986; Lee et al., 1990; Geiser, 1996; Mzilikazi and Lovegrove, 2002). It is possible therefore that the breeding pattern observed in *G. moholi* may have precluded the use of heterothermy. It is noteworthy that although the breeding pattern observed in *G. moholi* obviates torpor use, it allows for two litters during the summer (high reproductive output), which would presumably be adaptive if the animals are confronted with high predation risk.

In conclusion, although lesser bushbabies seemed excellent candidate employers of heterothermy, we found no evidence of torpor in 11 free-ranging animals between February 2002–August 2003. We suggest that the breeding pattern observed in *G. moholi* obviates torpor use in the species while increasing fecundity and that such a breeding pattern is likely to be adaptive in environments where predation pressure is high.

Acknowledgements

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The Origin of Mammalian Heterothermy: A Case for Perpetual Youth?

MICHAEL B. HARRIS,¹ LINK E. OLSON,^{1, 2} AND WILLIAM K. MILSOM³

¹ Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska, USA

² University of Alaska Museum, Fairbanks, Alaska, USA

³ Department of Zoology, University of British Columbia, Vancouver, BC, Canada

The phylogenetic distribution of heterothermic mammals is diverse, encompassing several independent lineages in which many heterotherms are closely related to nonheterotherms and in which there is no identifiable heterothermic common ancestor (Cade, 1964; Lyman, 1982; Nedergaard and Cannon, 1990; Malan, 1996; Geiser, 1998). The ability to utilize heterothermy, however, depends on a host of physiological specializations that appear common to all species that employ this strategy. The breadth and complexity of specializations underlying the phenotype, and the conservation of these specializations between species, argues against a polyphyletic origin for heterothermy and suggests that a capacity for heterothermy is a retained ancestral trait (Cade, 1964; Malan, 1996; Geiser, 1998).

It had been proposed that heterothermy represented an ancient characteristic found only in “primitive” mammals (Cade, 1964). The distribution of heterothermy, however, is not restricted to taxa traditionally considered “primitive” and includes placental mammals as well as marsupials and monotremes (Cade, 1964; Lyman, 1982; Nedergaard and Cannon, 1990; Malan, 1996; Geiser, 1998; Carey et al., 2003). One recent theory suggests that the physiological changes associated with sleep, which are common to all mammals, have been acted upon and extended in a polyphyletic fashion to produce the physiological changes associated with heterothermy (Berger, 1984; Kilduff et al., 1993).

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

Although an intriguing hypothesis, many characteristics shared by mammalian heterotherms are not related to sleep physiology.

However, remarkable similarity is apparent between the physiological characteristics associated with heterothermy and those associated with another common “ancestral trait,” neonatal life. In this article we argue that the capacity for heterothermy in adult mammals may have arisen multiple times in unrelated species through retention of heterothermic characteristics common to neonatal mammals: an example of evolutionary adaptation by neoteny. Heterothermy in adult mammals could be a paedomorphic trait, resulting from the retention of juvenile characteristics into adult life.

Although the ability to hibernate appears to involve unique patterns of gene expression, the phenotype has not been associated with novel genes (Srere et al., 1992; Carey et al., 2003). It would appear that the genes required to specify the hibernation phenotype are common to the mammalian genome. The hypothesis of “hibernator as neonate” would suggest that the genetic potential for heterothermy is expressed to some extent in all neonates and that heterothermy in adults results from the continued expression of such genes. This could even reflect the genetic potential to accommodate heterothermy in reptiles, conserved in the mammalian genome (Malan, 1996; Geiser, 1998).

We propose that in species where the adaptive benefits of heterothermy persisted beyond the neonatal period, this trait was retained into adulthood. This resulted in the continued expression of genes and gene products advantageous to the neonate for facilitating heterothermy in adults. Variations in the conserved expression of this potential provide the basis for the range of heterothermic phenotypes spanning from facultative torpor to shallow and deep torpor and seasonal hibernation.

In support of this hypothesis are the profound similarities between many general physiological characteristics of neonates and specific “adaptations” of heterothermic mammals. The following represent examples, taken from a range of systems, intended to illustrate the diversity of adaptations common to heterothermic mammals that could have originated from neonatal physiology.

Reduced Body Temperature and Metabolic Suppression

Most neonatal mammals can tolerate extreme reductions in body temperature compared to their adult counterparts (Guignard and Gillieron, 1997). This goes beyond mere tolerance, however, and orchestrated reductions in metabolism and body temperature are relatively common neonatal traits. Thus, endogenous

torpor-like variations in core temperature and metabolism are well documented in many neonatal rodents (Nuesslein-Hildesheim et al., 1995).

Tolerance to Hypoxia, Ischemia, and Asphyxia

During hibernation and torpor, heterothermic mammals appear to suffer no ill effects from prolonged hypoxia, asphyxia, or ischemia. This is also true of euthermic heterotherms, and isolated tissues from heterotherms, but not of mature nonheterotherms or their tissues (Frerichs and Hallenbeck, 1998, see Drew et al., in press, for review). Such tolerance is also seen in mammalian neonates, but is not retained in adults of nonheterotherms (Frerichs, 1999; Singer, 1999; Wagner et al., 1999; Zhou et al. 2001; Drew et al., in press).

Brown Adipose Tissues (BAT)

BAT is essential for nonshivering thermogenesis and rapid warming during arousal from torpor in heterothermic placental mammals. Pronounced BAT deposits are so universal and conserved in heterothermic placental mammals that they have been considered a deterministic feature described as a “hibernation gland” (Cade, 1964). Similar pronounced BAT deposits serve the same role in rapid thermogenesis in mammalian neonates. In most nonheterothermic mammals BAT is greatly reduced or lost during maturation. Incidentally, although heterothermy occurs both in mammals and birds, homology between the two has been questioned because of the absence of BAT in birds (discussed in Geiser, 1998; Geiser and Ruf, 1995). Heterothermy in birds and mammals may yet be homologous, however, if it developed through the retention of the heterothermic capacity shared by the neonates of each group, with the independent origin of BAT in neonatal placental mammals and not in neonatal birds. The limited role of BAT in heterothermic monotremes and marsupials may be similarly explained.

White Adipose Tissues, Fat Metabolism, and Lipogenesis

The metabolism of the mammalian neonate is acutely refined to convert a high-fat diet into white adipose tissue, and nonheterotherms generally show age-related decreases in this capacity for lipogenesis. Seasonal hibernators, however, maintain the ability to build fat rapidly in the fall and, in species that do not store food, to fuel metabolism primarily from stored fat during hibernation (Nedergaard and Cannon, 1990; Carey et al., 2003).

Digestive System Plasticity

During the postnatal period mammals switch from a prenatal strategy, where nutrients are supplied to the fetal blood via the umbilical circulation, to autonomous nutrient uptake requiring ingestion, digestion, and absorption of nutrients across the gut epithelium. This switch necessitates rapid growth in the neonatal digestive system. During prolonged torpor, the digestive system atrophies, and is rapidly rebuilt on arousal (Carey, 1990). This rapid regrowth of the digestive system could reflect processes similar to those occurring in the neonate.

Autonomic Nervous System

Cardiac Sympathetic Innervation

The heart is influenced by both sympathetic and parasympathetic branches of the autonomic nervous system. Parasympathetic innervation is established *in utero*, while cardiac sympathetic innervation develops during the neonatal period in most mammals (Kralios and Kralios, 1996; Johansson, 1996; Wang and Zhou, 1999). Cardiac sympathetic innervation appears to be diminished or absent in mammalian hibernators (Neilsen and Owman, 1968). In both cases, this is proposed to be an adaptation designed to limit cardiac fibrillation.

Vagal Afferent Integration

Breathing is generated by the brainstem but is shaped by respiratory reflexes involving feedback from a host of receptors, including afferent projections of the vagus nerve responding to lung stretch. The magnitude of many such reflexes decrease with age. When euthermic, however, some heterothermic rodents demonstrate an acute reliance on vagal feedback unlike other mature mammals but similar to the situation observed in neonates (Fedorko et al., 1988; Harris and Milsom, 2001).

Neuronal Growth and Plasticity

Heterotherms demonstrate distinct reductions in the complexity of synapses during torpor and remarkable synaptic expansion on arousal (Popov et al., 1992). Heterotherms also demonstrate greater tolerances to and recovery from brain injury (reviewed in Zhou et al., 2001). These are traits that strongly mimic the neural plasticity of the neonatal period.

Pulmonary Surfactant

Surfactant controls the surface tension of the fluid lining the lung. Homeothermic mammals experience pulmonary surfactant dysfunction with relatively small fluctuations in body temperature, while the surfactant systems of neonates and heterotherms function over a wide range of temperatures (Ormond et al., 2003; Slocombe et al., 2000). Surfactant composition changes during postnatal development and in heterotherms in and out of torpor (Ballard et al., 2003; Slocombe et al., 2000).

Haemoglobin

The oxygen-carrying capacity of the blood of heterotherms is enhanced relative to that of nonheterotherms (Maginniss and Milsom, 1994). Furthermore, in heterotherms, the oxygen affinity of the oxygen-binding protein haemoglobin is greater than that found in nonheterotherm haemoglobins. Haemoglobin is also different in the fetus and the adult, with fetal haemoglobin having a higher affinity for oxygen. Postnatal maturation is associated with a gradual replacement of fetal haemoglobin with the lower affinity, adult form (Halleux et al., 2002).

An Example from the Tenrecidae

Much of the theory surrounding the origins of mammalian heterothermy has dealt with a limited subset of living mammals. Most data come from rodents, marsupials, and monotremes (Cade, 1964; Lyman, 1982; Nedergaard and Cannon, 1990; Malan, 1996; Geiser, 1998; Carey et al., 2003). Identifying closely related heterothermic and nonheterothermic species with which to test theories of the origins of heterothermy is difficult. Here we provide such an example, in a group that has thus far received relatively little attention from the physiological community. The mammalian family Tenrecidae includes three species restricted to equatorial Africa and 26 currently recognized species on the island of Madagascar (Jenkins, in press). The Malagasy tenrecs represent a spectacular example of an island radiation resulting from a single colonizing common ancestor (Olson and Goodman, 2003). In addition to the striking level of interspecific variation in morphology, ecology, life history, and behavior found in Malagasy tenrecs (reviewed in Olson, 1999, and Olson and Goodman, 2003), there is a range of homeothermic capacities across the clade (reviewed in Racey and Stephenson, 1996). This range includes species that fail to show homeothermy at any temperature (*Geogale aurita*), others that utilize daily or seasonal torpor (*Tenrec ecaudatus*, *Hemicentetes nigriceps*, *H. semispinosus*) and still oth-

ers that maintain relatively constant body temperatures (*Microgale cowani*, *M. melanorrhachis*) (Stephenson, 1991; Racey and Stephenson, 1996). Of particular interest in the current context are the spiny tenrecs (subfamily Tenrecinae). Molecular and morphological data strongly support a sister relationship between two species in this five-member clade, the lesser (*Echinops telfairi*) and greater (*Setifer setosus*) Malagasy hedgehog tenrecs. While *Echinops* and *Setifer* are very similar externally, subtle morphological differences between the two (reviewed in Olson, 1999) suggest that several features in *Echinops* may be paedomorphic (neotenus) relative to its closest living relative, *Setifer*. These features include differences in body size—adults of *Setifer* tend to be much larger than those of *Echinops* (Garbutt, 1999)—and, in *Echinops*, a failure to develop a third molar (Thomas, 1892). *Echinops* is the only member of the family that lacks this tooth, which is the last of the three molars to erupt in all other species.

Physiologically, *Echinops* is striking in its inability to maintain a constant body temperature (T_b) across a broad range of ambient temperatures (T_a) (Nicoll and Thompson, 1987; reviewed in Stephenson, 1991) and is considered to be a true heterotherm except during periods of gestation and lactation (see Poppitt et al., 1994). Field and captive studies on *Setifer*, on the other hand, have demonstrated its ability to maintain a T_b higher than T_a throughout its annual cycle (Eisenberg and Gould, 1970) and, thus, *Setifer* is classified as being more homeothermic than *Echinops*. Both species are known to enter daily and seasonal torpor, but torpor in *Setifer* is believed to be less profound than in *Echinops*. (Eisenberg and Gould, 1970; Stephenson, 1991; Salton and Buffenstein, 2004).

While we would clearly not want to base our hypothesis on a single empirical example, we offer the case of *Echinops* and *Setifer* as evidence of a possible relationship between paedomorphosis and relative heterothermy.

Conclusion

There is remarkable similarity between traits underlying the heterothermic phenotype and traits common to neonatal mammals. Thus, we propose that mammalian heterothermy is an example of evolution through neoteny and that the seemingly polyphyletic distribution of heterothermy in mammals (and perhaps also birds) results from independent cases of arrested physiological development in lineages where the preservation of heterothermic capacity in adults conveyed a selective advantage. The conservation of complex traits in distantly related heterotherms reflects a common origin in neonatal physiology. This theory provides a basis for testable hypotheses, and would predict trait similarities between

neonates of heterothermic and nonheterothermic sister species, and a retention of traits during maturation in heterotherms but a loss in nonheterotherms. This theory also predicts that when one species is more heterothermic with respect to its sister species, it will also be relatively paedomorphic. Answers to previously enigmatic questions pertaining to adaptations for torpor and hibernation could be sought through comparison with neonates. Similarly, insights into neonatal physiology and certain pathophysiology might be gained through investigation of heterothermic mammals.

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Passive Rewarming from Torpor in Mammals and Birds: Energetic, Ecological and Evolutionary Implications

FRITZ GEISER,¹ REBECCA L. DRURY,¹ GERHARD KÖRTNER,¹ CHRISTOPHER TURBILL,¹ CHRIS R. PAVEY,² AND R. MARK BRIGHAM³

¹ Centre for Behavioural and Physiological Ecology, Zoology, University of New England, Armidale NSW 2351, Australia

² Parks and Wildlife Service NT, PO Box 2130, Alice Springs 0871, Australia

³ Department of Biology, University of Regina, Regina SK S4S 0A2, Canada

Abstract. Endothermic rewarming from torpor requires an enormous increase in energy expenditure and is widely viewed as one of the major disadvantages of torpor. However, recent evidence suggests that passive rewarming, by the increase of ambient temperature (T_a), by basking in the sun, or by social thermoregulation, is common in heterothermic birds and mammals. Passive rewarming has been observed in a number of mammals, including echidnas, several dasyurid marsupials, pygmy possums, marmots, elephant shrews, lemurs, several insectivorous bats, and possibly numbats and sugar gliders. In birds, passive rewarming occurs in poor-wills, whip-poor-wills, Australian owl-nightjars, tawny frogmouths, and roadrunners. Passive rewarming is employed by both hibernators (species capable of prolonged torpor) and daily heterotherms (species displaying exclusively daily torpor), presumably to reduce energy expenditure during arousal. In captivity, passive rewarming due to increased T_a reduced energy expenditure of a small marsupial during rewarming by ~65% and, with a radiant heat source, by ~85% of that required for active, endothermic rewarming. We estimate that the use of daily torpor, combined with passive rewarming from torpor, and basking in the sun during the rest phase can reduce daily energy expenditure by ~50%. The low energy expenditure required during passive rewarming may explain how ancestral endotherms with presumably a low capacity

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for heat production were able to rewarm from low body temperatures (T_b) before they commenced activity. In addition, it may explain why torpor is more common in sunny regions or in regions with daily T_a fluctuations including T_a maxima $> T_b$ minima during torpor. As a consequence, many other species that have access to sun or high daily fluctuations of T_a may use torpor because they can save energy during torpor and arouse at minimal rewarming costs via passive rewarming and do not require a high thermogenic capacity.

Introduction

It is widely accepted that torpor in mammals and birds, which is characterised by periodic reductions of body temperature (T_b) and metabolic rate (MR), is primarily used for energy conservation (Lyman et al., 1982; Boyer and Barnes, 1999). Nevertheless, a major drawback to this physiological strategy is the high expenditure of energy that is required for endothermic arousals at the end of a torpor bout. This is especially true for short bouts of torpor such as during daily torpor, which usually lasts < 12 hours within a 24-hour cycle. Although MR may be reduced by $\sim 90\%$ during a bout of daily torpor compared with that for normothermic, resting individuals, daily energy savings through the use of torpor are usually only $\sim 20\text{--}30\%$ because of the high costs of activity and rewarming (Kenagy, 1989; Holloway and Geiser, 1995; Lovegrove et al., 1999). Even during hibernation, when MR during torpor (TMR) may be $< 1\%$ of that in normothermic individuals (Geiser, 2004), endothermic arousals that occur after torpor periods of several days or weeks require most of the energy used during the hibernation season (Wang, 1978; Thomas et al., 1990).

Estimates of energy expenditure during torpor have largely been based on laboratory data obtained under constant ambient temperature (T_a). However, recent evidence from field studies in both mammals and birds demonstrates that rewarming in many species is to a large extent a passive process (Schmid, 1996; Körtner et al., 2000; Brigham et al., 2000; Dausmann et al., 2000; Geiser et al., 2002; Mzilikazi et al., 2002; Geiser, 2003; Lane et al., 2004; McKechnie and Wolf, 2004). For captive animals, rewarming costs can be reduced significantly by passive rewarming associated with a rise in T_a (Lovegrove et al., 1999), by exposure to radiant heat (Geiser and Drury, 2003), or by social thermoregulation (Arnold 1993). Consequently, the energetic costs of rewarming in many species may have been substantially over-estimated in the past and the potential energetic benefits of torpor in free-ranging animals under-estimated.

In this paper we summarise current knowledge about passive rewarming by heterothermic mammals and birds and how passive rewarming may reduce energy expenditure.

Passive Rewarming in Mammals and Birds

Passive rewarming from torpor appears to be widespread in both mammals and birds (Table 1). It occurs in all three mammalian subclasses, but in birds is currently known only from the Caprimulgiformes and roadrunners, although it is likely that other birds (e.g., mouse birds or hummingbirds) use exogenous heat in the rewarming process. Passive rewarming occurs in both hibernators and daily heterotherms. Passive rewarming has been observed from $T_b < 10^\circ \text{C}$ in some hibernators, but usually begins at $T_b \sim 20^\circ \text{C}$. In most species passive rewarming does not appear to involve movement, although it may involve active selection of an appropriate torpor site in which to rewarm passively on the following day. However, active movement at low T_b from a torpor site to a favourable basking site has been observed in fat-tailed antechinus and tawny frogmouths. In species with social thermoregulation, such as marmots or sugar gliders, passive rewarming may simply involve delayed arousal and absorbing heat from adjacent individuals. Most observations on passive rewarming have been made recently, reflecting the greater use of small temperature transmitters and data loggers in the field. Thus it is likely that passive rewarming will be reported in many other heterothermic species when these are investigated in nature.

For those species for which MR measurements are available, MR remained low (below or near basal MR, BMR) during the initial part of the rewarming process. However, in species in which high or rising T_a caused the increase of T_b , endothermic arousal associated with a sharp rise of MR commenced when T_b reached $\sim 25^\circ \text{C}$. It has been estimated that passive rewarming via an increase in T_a from 15 to 25°C reduces the rewarming costs in dunnarts (*Sminthopsis macroura*) to $\sim 35\%$ relative to active rewarming (Lovegrove et al., 1999). During radiant heat assisted passive rewarming in *S. macroura* basking under a heat lamp, which provided much less radiant heat than the sun, endothermic heat production remained low (below BMR) throughout most of the rewarming process, without the sharp rise near $T_b \sim 25^\circ \text{C}$. As a consequence, radiant heat assisted passive rewarming reduced rewarming costs by *S. macroura* to $\sim 15\%$ of that required for active endothermic rewarming at the same T_a (Geiser and Drury, 2003).

Table 1. Observations on passive rewarming from torpor in mammals and birds.

	Body mass (g)	Observations	Source
MAMMALS			
Monotremata			
<i>Tachyglossus aculeatus</i> Echidna	~4000	Partially passive rewarming in free-ranging individual in a burrow in spring.	Brice et al. 2002
Marsupialia			
<i>Planigale tenuirostris</i> Narrow-nosed planigale	7	Basks in winter, but not clear whether during or after rewarming from torpor.	Read 1995a
<i>Planigale gilesi</i> Giles' planigale	8	Displays daily torpor in captivity and basks in winter in the wild, but not clear whether during or after rewarming.	Read 1995b; Geiser 2003
<i>Sminthopsis murina</i> Common dunnart	16	Partially passive rewarming with T_a in the field from T_b ~20° C.	Paull 2004
<i>Sminthopsis macroura</i> Stripe-faced dunnart	25	Has been observed basking in captivity; individuals reduce energetic cost of rewarming from torpor to ~35% of endothermic rewarming when exposed to rise of T_a , and to ~15% endothermic rewarming when exposed to radiant heat.	Lovegrove et al. 1999; Geiser & Drury 2003
<i>Pseudantechinus macdonnellensis</i> Fat-tailed antechinus	31	Torpid individuals emerge from deep rock crevices to commence morning basking at T_b 19.3 to 31.3° C; remain in sun throughout rewarming process and for much of the afternoon.	Geiser et al. 2002
<i>Dasyercus cristicauda</i> Mulgara	110	Displays daily torpor in captivity and basks in the wild, but not clear whether during or after rewarming from torpor.	Woolley 1995; Geiser 2003
<i>Myrmecobius fasciatus</i> Numbat	500	Observed basking in the morning, but not clear whether during or after rewarming from torpor.	Serventy & Raymond 1973
<i>Cercartetus concinnus</i> Western pygmy-possum	18	Partially passive rewarming of free-ranging individual in the morning from T_b ~9 to 17° C.	Geiser & Körtner 2004

<i>Petaurus breviceps</i> Sugar glider	100	Synchronised arousals among individuals sharing a nest; a slight delay in onset of arousal will result in partially passive rewarming in that individual.	Körtner & Geiser 2000
Placentalia			
<i>Marmota marmota</i> Alpine marmot	~3000	Synchronised endogenous arousals reduce energy expenditure during periodic arousals; adults provide heat for juveniles.	Arnold 1993
<i>Elephantulus myurus</i> Rock elephant shrew	56	Short bouts of torpor with $T_b < 10^\circ \text{C}$ common in captivity and field, but occasional bouts of $> 1\text{d}$. Most bouts in the field terminated within 2 hour after sunrise, T_a and T_b rise are tightly coupled, and basking may be used during rewarming.	Lovegrove et al. 2001; Mzilikazi et al. 2002
<i>Eremitalpa granti</i> Namib golden mole	20	Displays daily variations of T_b from $\sim 20^\circ \text{C}$ in the morning to $\sim 30^\circ \text{C}$ in the afternoon tracking sand temperature.	Fielden et al. 1990
<i>Microcebus myoxinus</i> Pygmy mouse lemur	33	Passive rewarming with T_a in outdoor enclosure. T_b rises from ~ 19 to 27°C with small rise of MR.	Schmid 1996
<i>Microcebus murinus</i> Grey mouse lemur	80	Passive rewarming with T_a in outdoor enclosure. T_b rises from ~ 18 to 26°C and MR increases by less than 2-fold. Endothermic arousal at $T_b > 26^\circ \text{C}$.	Schmid 1996
<i>Cheirogaleus medius</i> Fat-tailed lemur	250	Hibernates for several months in dry, warm winter of Madagascar, but daily partial arousals from $T_b \sim 20$ to $\sim 30^\circ \text{C}$. T_b rise to $\sim 27^\circ \text{C}$ is largely passive, at higher T_b active heat production commences with $\text{MR} > \text{BMR}$.	Dausmann et al. 2000
<i>Vespadelus pumilus</i> Eastern forest bat	4	Partial passive arousal in the morning in a subtropical area in summer with an increase of T_b from ~ 16 to 23°C in parallel with rising T_a .	Turbill et al. 2003a

<i>Myotis lucifugus</i> Little brown bat	6	Partially passive rewarming from T_b ~18–23° C via increasing T_a under simulated roost conditions; MR remains low until T_b ~25–28° C is reached and increases steeply thereafter.	Kurta 1990
<i>Myotis evotis</i> Western long-eared bat	7	Partially passive rewarming of a pregnant bat from T_b ~19 to 30° C.	Chruszcz & Barclay 2002
<i>Nyctophilus geoffroyi</i> Australian long-eared bat	7	Prolonged torpor bouts of up to 2 weeks in winter, daily passive rewarming from T_b ~2–8 to 10–20° C, active arousals after 1–2 weeks in torpor with T_b > 30° C. In summer partially passive arousal near midday on most days.	Turbill et al. 2003b, c
<i>Eptesicus fuscus</i> Big brown bat	20	Passive rise of T_b with T_a from T_b ~10° C in outdoor enclosure; partially passive rewarming in free-ranging bat.	Lausen & Barclay 2003; Willis & Brigham 2003

BIRDS

Caprimulgiformes

<i>Phalaenoptilus nuttallii</i> Poor-will	45	Prolonged and deep torpor (T_b < 10° C) in winter, daily partially passive arousals due to solar radiation; endogenous arousal after ~5 days in shaded birds.	Brigham et al. 2004
<i>Caprimulgus vociferus</i> Whip-poor-will	55	Rise of T_b with T_a from T_b ~20 to 30° C in late spring.	Lane et al. 2004
<i>Aegotheles cristatus</i> Owlet-nightjar	50	Dawn torpor in winter common, arousal near midday when birds were observed basking.	Brigham et al. 2000
<i>Podargus strigoides</i> Tawny frogmouth	500	Nocturnal torpor on cold winter nights, arousal near sunrise; second dawn torpor bout commonly terminated by partially passive rewarming in the sun.	Körtner et al. 2000, 2001

Cuculiformes

<i>Geococcyx californicus</i> Roadrunner	300	Passive rewarming in mildly hypothermic birds from T_b ~34 to 38° C under artificial sunlight in captivity.	Ohmart & Lasiewski 1971
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The potential effect of radiant energy on daily energy expenditure by *S. macroura* is substantial (Fig. 1). To estimate daily energy expenditure we assume both passive rewarming from torpor as well as basking during normothermia, which is common in both mammals and birds, and allows a reduction of energy expenditure to \sim BMR over a wide range of T_a . We calculate that energy expenditure of individuals remaining normothermic throughout the day with an activity phase at night and rest phase during the daytime is 48 kJ/d (average from 6

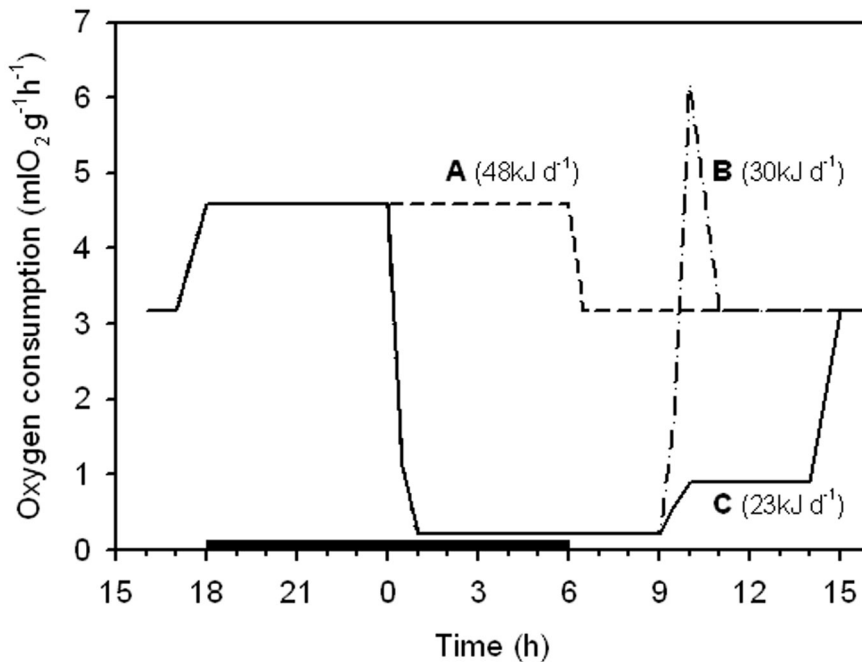


Fig. 1. Time course of metabolic rate measured as oxygen consumption of *Sminthopsis macroura* (25 g) exposed to T_a 16° C with or without access to radiant heat. Heat was provided by a 60W reflector globe with 99% color rendering index and 2850K, which produced \sim 5% of the lux of natural solar radiation. (A) Animals without access to radiant heat remaining normothermic throughout (active night, rest day), (B) animals entering torpor at midnight without access to radiant heat and endothermic arousal from 09:00h, and (C) animals entering torpor at midnight and having access to radiant heat during rewarming and for most of the afternoon, assuming that during basking in the sun MR falls to near BMR as during basking under a 60W reflector globe. The black bar on the x-axis indicates the dark phase (MR data based on six individuals from Geiser and Drury 2003).

individuals held at T_a 16° C without access to radiant heat). If animals entered torpor at midnight as commonly observed and used endothermic arousal, daily energy expenditure is reduced by 37%. However, if they used passive rewarming in the morning and basked for most of the afternoon, daily energy expenditure is reduced by 52%. These calculations indicate that access to solar energy during passive rewarming from torpor and during the normothermic rest phase, combined with low TMR, enable animals to benefit more substantially from torpor than previously thought.

Passive rewarming also has implications for torpor use. Access to solar radiation and/or exposure to daily T_a fluctuations, including T_a maxima > T_b minima during torpor may be important factors that determine whether or not a species uses torpor, in addition to unpredictable changes in climate and food availability that often are associated with daily torpor (Lovegrove, 2000). Most species in Table 1 live in areas that receive substantial amounts of solar radiation or experience pronounced daily T_a fluctuations such as deserts and high altitudes, which may be limiting with respect to food availability but provide alternative energy in the form of sunshine. Because moderate, changing T_a and basking can substantially reduce arousal costs and because species diversity increases towards the equator in most taxa, it is likely that the number of heterothermic endotherms living at low latitudes may have been underestimated in the past. Thus, our summary challenges the traditional view that torpor is especially common in cold climates.

We speculate that passive rewarming was probably also involved in the evolution of endothermic thermoregulation. The >10-fold increase in thermogenic capacity from ectotherms to endotherms (Hulbert and Else, 1989) must have involved numerous generations of individuals with intermediate thermogenesis. Ancestral endotherms were small, likely unable to maintain a constant high T_b during rest, and lacked the thermogenic capacity required for endothermic rewarming, but probably relied on high T_b s for efficient function. If passive rewarming reduced energy expenditure during the rewarming phase by 65 to 85% of that required for active rewarming, a small thermogenic capacity would have sufficed to raise T_b to levels that allowed efficient function.

Our observations suggest that ancestral endotherms were heterothermic and became torpid when thermally challenged or when food supply was low (Johansen, 1961; Cade, 1964; Grigg and Beard, 2000). However, it is improbable that the pattern of torpor was identical to that in modern heterotherms with thermoregulatory control of T_b during torpor and the capacity for full endother-

mic arousal. It therefore seems likely that ancestral endotherms required external heat, not primarily to save energy as in extant species, but either to speed up the rewarming process or be able to rewarm at all.

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Solar Radiation and the Energetic Cost of Rewarming from Torpor

ANDREW E. MCKECHNIE AND BLAIR O. WOLF

Biology Department, MSC03-2020, 1 University of New Mexico, Albuquerque, NM 87131-0001, USA

Abstract. We modeled the effect of basking behavior on the energy required to rewarm from torpor (E_{rewarm}), using solar heat gain data for birds. Our model suggests that reductions in E_{rewarm} can be considerable (up to ca. 60%), and vary with latitude, season, and time of day. We argue that variation in the effects of solar radiation (SR) on rewarming rate and E_{rewarm} can be thought of as a continuum from passive to augmented modes of SR-assisted rewarming. Passive rewarming rates are generally lower than endogenous rewarming rates.

Introduction

The energy required to rewarm to normothermic body temperature is a major constraint on the energetic benefits of torpor (Prothero and Jürgens, 1986; McKechnie and Wolf, this volume). Short-wave solar radiation can dramatically affect the energy balance of endotherms, and there has recently been considerable interest in the importance of solar radiation (SR) for ameliorating the energetic cost of rewarming. For example, Mzilikazi et al. (2002) provided indirect evidence that the majority of arousals in free-ranging rock elephant shrews (*Elephantulus myurus*) were SR-assisted. Geiser et al. (2002) found that fat-tailed antechinus (*Pseudantechinus macdonnellensis*) bask extensively during rewarming. Less is known from birds, but solar radiation appears to be involved in rewarming from shallow hypothermia in greater roadrunners (*Geococcyx californianus*), torpor in tawny frogmouths (*Podargus strigoides*), and hibernation in common

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poorwills (*Phalaenoptilus nuttalli*) (Ohmart and Lasiewski, 1971; Körtner et al., 2000; Woods, 2002).

The heat loads experienced by endotherms during exposure to solar radiation depend on numerous factors. Various structural and optical properties of the pelage or plumage interact with properties of the physical environment (irradiance and wind speed) to determine the heat load transferred to the skin (Walsberg, 1983; Wolf and Walsberg, 2000). Hence, it is impossible to model in a simple way the rate of heat transfer during basking behavior. However, laboratory measurements of solar heat gain (SHG), calculated as the reduction in metabolic heat production during exposure to simulated solar radiation (Wolf et al., 2000), can be used to estimate these heat fluxes under natural conditions. We developed a model based on avian SHG data, in order to investigate the potential effects of solar radiation on the energetics of rewarming from torpor.

Materials and Methods

To place boundaries on the solar radiation potentially available to endotherms, we calculated annual maximum and minimum levels for various latitudes. We predicted solar irradiance at an altitude of 600 m a.s.l. at various times of the day at various latitudes using equations 11.1 to 11.13 in Campbell and Norman (1998), assuming an atmospheric transmittance of 0.65. We then obtained SHG data collected or estimated for free convective conditions from laboratory studies for white-crowned sparrows (*Zonotrichia leucophrys*; De Jong, 1976; Wolf et al., 2000), greater roadrunners (*Geococcyx californianus*; Ohmart and Lasiewski, 1971), brown-headed cowbirds (*Molothrus ater*), zebra finches (*Taeniopygia guttata*; Lustick, 1969), and white-backed mousebirds (*Colius colius*; McKechnie, 1998). Because SHG is affected by an animal's orientation, we calculated solar heat gain per unit projected area perpendicular to the irradiance beam (A_p). In studies where the orientation of the bird relative to the irradiance source was not specified, we assumed a ratio of A_p to total surface area (A_p/A) of 0.24, corresponding to a prolate spheroid with a major axis twice as long as the minor axis, orientated at 60° to the irradiance beam (Campbell and Norman, 1998; Wolf et al., 2000). We used these data to generate an equation relating SHG to irradiance. We then used this equation to estimate SHG from predicted solar irradiance. To estimate the energy savings associated with basking during rewarming from torpor, we integrated predicted SHG and compared it to the estimated energetic cost of rewarming (E_{rewarm}) at an air temperature of 0°C and a torpor body temperature setpoint of 18°C (McKechnie and Wolf, this volume). We

assumed (1) that a bird is exposed to solar irradiance for the entire duration of the rewarming phase, and (2) that rewarming rate during SR-assisted rewarming is identical to that during endogenous (i.e., non-SR-assisted) rewarming. Hence, this model examines only one of several ways in which solar radiation can affect E_{rewarm} (see Discussion).

Results

Solar irradiance shows considerable variation with time of day, time of year, and latitude. Seasonal variation is small at low latitudes but increases at higher latitudes. At the equator, solar irradiance one hour after sunrise varies from a minimum of 366 W m^{-2} (solstice) to a maximum of 403 W m^{-2} (equinox), and at midday varies from $1,011 \text{ W m}^{-2}$ (solstice) to $1,046 \text{ W m}^{-2}$ (equinox). At a latitude of 30° , solar irradiance one hour after sunrise varies from 240 W m^{-2} (winter solstice) to 285 W m^{-2} (summer solstice), and at midday varies from 813 W m^{-2} (winter solstice) to $1,043 \text{ W m}^{-2}$ (summer solstice). At a latitude of 60° , solar irradiance on the summer solstice is 61 W m^{-2} one hour after sunrise and 955 W m^{-2} at midday, but never increases above 90 W m^{-2} during the 5.4 hour long day on the winter solstice.

The SHG and irradiance data we obtained from the literature were best described by a linear regression forced through the origin, with $\text{SHG} = 0.2055$ irradiance ($r^2 = 0.463$), where SHG and irradiance are in W m^{-2} . The fact that a linear regression model provided a better fit than an exponential decay model (De Jong, 1976; Walsberg et al., 1997) probably reflects variation in experimental conditions, such as lamps with different color temperatures, typically well below that of the sun.

Our model predicts that birds save considerable amounts of energy by basking during rewarming from torpor (Fig. 1). Our model also reveals that the energetic benefits of SR-assisted rewarming vary with latitude, season, and body size but also with the time of day when rewarming occurs (Fig. 1). For instance, low irradiance levels early in the day mean that small birds rewarming from torpor immediately following sunrise derive very little benefit from SR-assisted rewarming (Fig. 1). In fact, over most of the body size range we examined, SR-assisted rewarming is more beneficial for species that rewarm later in the day, since rewarming then coincides with higher irradiance levels (Fig. 1).

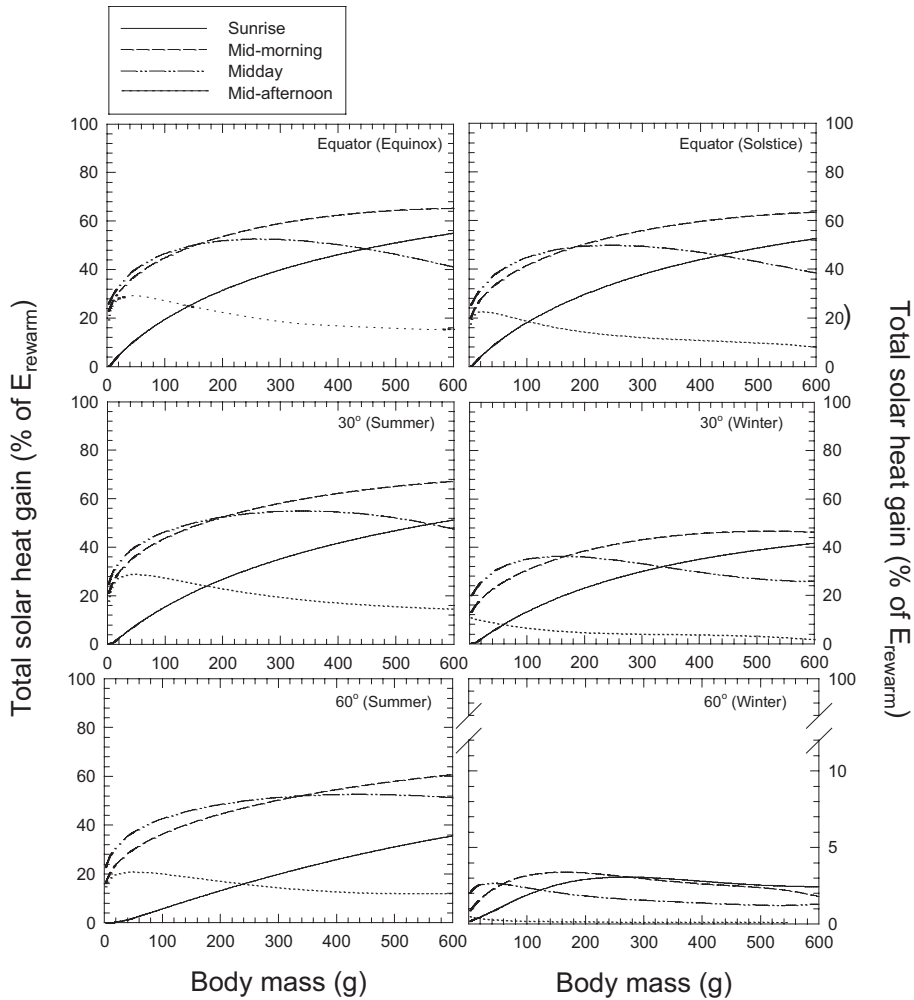


Fig. 1. Predicted avian energy savings associated with SR-assisted rearming from torpor as a function of body mass. The savings are predicted for maximum (left-hand graphs) and minimum (right-hand graphs) annual solar irradiance levels at three latitudes: equator (top graphs), 30° C (center graphs) and 60° C (bottom graphs). In each graph, energy savings are predicted for birds commencing rearming from torpor at sunrise, midmorning, midday, and midafternoon respectively.

Discussion

Our model predicts that basking behavior substantially reduces energy expenditure during rewarming and suggests that the availability of solar radiation is an important determinant of the energetic benefits of torpor. These predictions are consistent with several recent studies that examined the importance of basking behavior in free-ranging endotherms (Geiser et al., 2002; Mzilikazi et al., 2002; Woods, 2002). Geiser et al. (2002) found that *P. macdonnellensis* adjust the timing of rewarming to coincide with the availability of solar radiation. On clear days, *P. macdonnellensis* typically delayed rewarming until >2 hours after sunrise (Geiser et al., 2002). Mammalian data are very similar to those for birds in terms of the percentage of solar irradiance that represents physiologically significant SHG, suggesting that our model's predictions are applicable to both mammals and birds.

In striped-faced dunnarts (*Sminthopsis macroura*), E_{rewarm} during radiant energy-assisted rewarming was reduced by 85% compared to endogenous arousal (Geiser and Drury 2003), a greater reduction than predicted by our model (Fig. 1). These authors used a radiant heat source with a color temperature of 2850° K, substantially lower than that of the sun (5800° K). Animals generally absorb a far greater fraction of intercepted long-wave radiation than short-wave radiation (Walsberg 1983; Wolf and Walsberg 2000). Hence, the large reductions in E_{rewarm} observed by Geiser and Drury (2003) may in part reflect the fact that compared to the sun, the emission spectrum of their artificial radiation source was shifted towards longer wavelengths.

Solar radiation can affect the energetic cost of rewarming in several ways. At one extreme, endotherms may minimize E_{rewarm} by allowing solar radiation to passively elevate T_b . Passive rewarming will typically involve lower rewarming rates (see below) and lower energy expenditure than endogenous rewarming. At the other extreme, endotherms may minimize the duration of the rewarming phase by augmenting metabolic heat production with solar radiation. Augmented rewarming will involve higher rewarming rates and similar energy expenditure compared to endogenous rewarming. Passive and augmented modes of rewarming represent the ends of a continuum describing the potential effects of solar radiation on the energetic cost of rewarming from torpor. The scenario we have modeled in Figure 1, in which rewarming rates during SR-assisted rewarming remain the same as during endogenous rewarming, represents the midpoint. Mammalian data provide support for the notion of such a continuum. In rock elephant shrews, rewarming rates during SR-assisted rewarming were on

average < 33% of rewarming rates during endogenous rewarming (Mzilikazi et al., 2002), suggesting that rewarming was at least partly passive and hence that E_{rewarm} was reduced. In contrast, basking fat-tailed antechinus rewarmed twice as fast as nonbasking individuals (Geiser et al., 2002), suggesting augmented rewarming.

Rewarming rate should vary considerably depending on the mode of rewarming (passive SR-assisted, endogenous, or augmented SR-assisted). The theoretical maximum passive rewarming rate will occur when heat loss to the environment is zero, and under these conditions, $R_{\text{pmax}} = \text{SHG}/(sM_b)$, where R_{pmax} is the maximum passive rewarming rate ($^{\circ}\text{C min}^{-1}$), SHG is solar heat gain (J min^{-1}), s is the specific heat of animal tissues ($3.43 \text{ J g}^{-1} ^{\circ}\text{C}^{-1}$), and M_b is body mass (g). Predicted R_{pmax} is lower than endogenous rewarming rate, except in larger birds ($M_b > \text{ca. } 150 \text{ g}$) at high irradiance levels (Fig. 2). Because R_{pmax} is a theoretical maximum value possible only under unrealistic conditions of zero environmental heat loss, passive SR-assisted rewarming will almost always be slower than endogenous rewarming. The difference between R_{pmax} and endogenous rewarming rate increases with decreasing M_b (Fig. 2). Empirical observations support these predictions. Hibernating common poorwills (45 g) in artificially shaded roost sites rewarmed at an average of $0.39^{\circ}\text{C min}^{-1}$, but in sunlit roost sites passive warming occurred at ca. $0.08^{\circ}\text{C min}^{-1}$ (Woods, 2002).

One might expect differences in the extent of passive vs. augmented SR-assisted rewarming to be related to the need for energy conservation and to predation risk during rewarming. For instance, an endotherm that experiences a high predation risk might employ augmented SR-assisted rewarming to minimize the duration of the rewarming phase. On the other hand, an endotherm that experiences a low predation risk might employ passive SR-assisted rewarming to minimize E_{rewarm} , particularly in an environment where energy availability is low and/or unpredictable.

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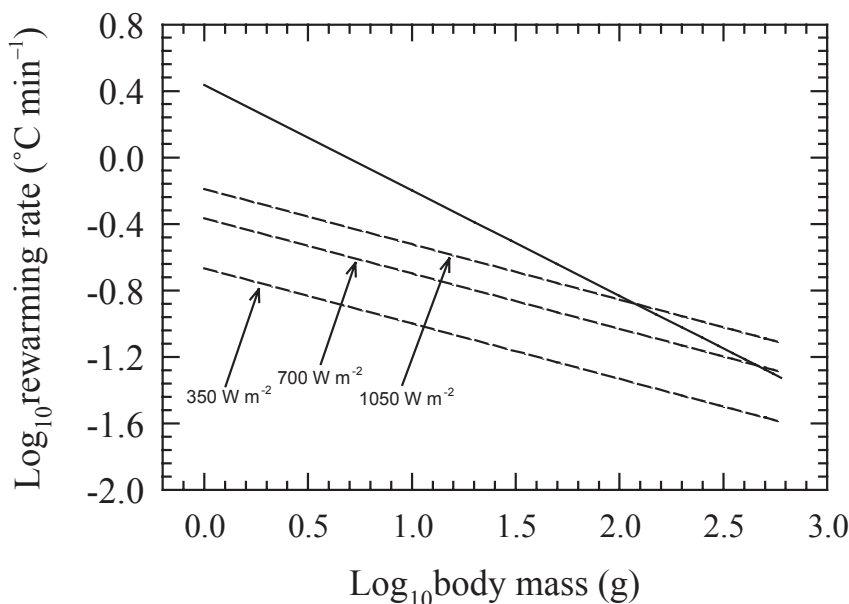


Fig. 2. Predicted avian maximum rewarming rate (R_{pmax}) during passive SR-assisted rewarming as a function of body mass. The dashed lines indicate R_{pmax} for three irradiance levels, covering the range of solar irradiance likely to be experienced by free-ranging endotherms. The solid line indicates predicted endogenous rewarming rate (McKechnie and Wolf, this volume).

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The Role of α -Linolenic Acid (18:3) in Mammalian Torpor

CRAIG L. FRANK,¹ WENDY R. HOOD,² AND MARY C. DONNELLY³

^{1,3} Fordham University, Louis Calder Center, Armonk, New York, USA

² Coastal Carolina University, Department of Biology, Conway, South Carolina, USA

Abstract. Field studies were conducted with free-ranging golden-mantled ground squirrels (*Spermophilus lateralis*) to determine the relationship between fall diet polyunsaturated fatty acid (PUFA) content, subsequent torpor patterns, and over-winter survival over a two-year period. Blood plasma PUFA levels increase with diet PUFA content. The PUFA contents of fall diets were thus estimated by measuring blood plasma PUFA levels. Torpor patterns and over-winter survival were measured by radio telemetry using temperature-sensitive collars. Mean plasma PUFA levels for juvenile *S. lateralis* were nearly twice those of adults, due to elevated linoleic acid (18:2) contents. One third of the juvenile *S. lateralis* did not survive the winter, whereas all of the adults survived this period. These findings indicate that the food plant selection of juvenile ground squirrels differs substantially from those of adults and produces a relatively higher PUFA intake. These results suggest that one consequence of juvenile dietary preferences may be a lowered over-winter survival rate through the inhibition of hibernation by high diet 18:2 contents, rather than due to differences in diet 18:3 levels.

Introduction

Laboratory experiments with chipmunks (*Tamias amoenus*), two species of ground squirrels (*Spermophilus lateralis* and *S. saturatus*), and marmots (*Marmota flaviventris*) have revealed that their torpor is enhanced when they ingest diets with moderately high levels of linoleic acid (18:2). Sciurids fed a moderately high linoleic acid diet were more likely to enter torpor, spent less time fasting

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prior to torpor, had lower metabolic rates, and had longer torpor bouts than those given diets containing relatively less linoleic acid (Florant et al., 1993; Frank, 1992; Geiser and Kenagy, 1987, 1993; Thorp et al., 1994). Similar experiments conducted with mice and 2 marsupials produced identical results (Geiser, 1991; Geiser et al., 1992; Withers et al., 1996). Torpor by golden-mantled ground squirrels (*Spermophilus lateralis*) is actually inhibited, however, when diet linoleic acid contents are above 62 mg/g (Frank and Storey, 1996), possibly due to enhanced lipid peroxidation (Frank and Storey, 1995), and is thus most likely when linoleic acid levels are 33–62 mg/g diet (Frank et al., 1998). Linoleic acid is a polyunsaturated fatty acid (PUFA) having more than one carbon-carbon double bond, as opposed to either a saturated fatty acid containing no carbon-carbon double bonds or a monounsaturated fatty acid containing only one such bond. Mammals can synthesize saturated and monounsaturated fatty acids, but they are incapable of producing PUFAs. Most plant species, however, produce two PUFAs: linoleic acid (18 carbon atoms, two double bonds) and α -linolenic acid (18 carbon atoms, three double bonds). When mammals consume PUFAs these are incorporated into their cell membranes and storage lipids (Gunstone, 1996).

Feeding by ground squirrels dramatically increases for two months prior to onset of hibernation, and a body fat content of 35–40% is attained (Kenagy, 1987; Kenagy and Barnes, 1988). The fall diet of ground squirrels consists mostly of plant tissues (Eshelmann and Jenkins, 1989; Kenagy et al., 1989; Tevis, 1953). The PUFA content of plants varies with species, season, and between different parts of the same plant (Florant et al., 1990; Gunstone, 1996). Alpha-linolenic acid (18:3) comprises $\frac{1}{3}$ to $\frac{1}{4}$ of all PUFAs found in the fall diets of herbivorous hibernators (Florant et al., 1990; Frank 1994), but little is known about the influence of it on torpor. Laboratory experiments using a 4.5% linseed oil diet as a source of α -linolenic acid suggested that this PUFA might actually inhibit torpor (Hill and Florant, 2000), although the authors indicated that this inhibition may have actually been due some compound(s) present in the linseed oil diet other than α -linolenic acid. We predict that: (1) a moderate level of α -linolenic acid in the diet actually enhances torpor, (2) a moderate intake of α -linolenic acid is maintained by mammalian hibernators through the selection of food items based on fatty acid composition, and (3) hibernator overwinter mortality is associated with exceedingly high diet PUFA contents during the previous fall. The first two hypotheses were tested in laboratory experiments involving *S. lateralis*, and the third was examined by a field study on this species.

Methods

A total of 28 adult *S. lateralis* were collected from the Crooked Creek (37° 30' N, 118° 10' W, elevation = 3094 m) area of the White Mountains in California during August 2000/2001. All were individually housed in rat cages located in the Wildlife Vivarium of Fordham University and maintained at 22–24° C on a natural fall (10L:14D) photoperiod. Three different semisynthetic diets varying only in fatty acid composition (Table 1) were produced: each used ground flax seed for an α -linolenic acid source. The low linoleic acid/low α -linolenic acid (L 18:2/L 18:3) diet was a mixture of 90% Purina 5001 Rodent Chow, 5% flax seed, and 5% coconut oil, the high linoleic acid/low α -linolenic acid (H 18:2/L 18:3) diet was composed of 90% Purina 5001 Rodent Chow, 5% flax seed, and 5% sunflower oil, and the low linoleic acid/high α -linolenic acid (L 18:2/H 18:3) diet consisted of 90% Purina 5001 rodent chow and 10% ground flax seed. The Test Diets Division of Purina Mills, Inc. produced the diets pressed into 1 g cylindrical pellets. All diets were 26% protein, 12% lipid, 51% carbohydrate, and 7.3% ash. Commercial food colorings were used to code for diet type. Color does not influence the diet selection of *S. lateralis* (Frank, 1994). Diet and blood plasma fatty acid compositions were determined by gas-liquid chromatography following the procedures summarized by Frank (2002).

Table 1. Fatty acid compositions of the semisynthetic diets.

Fatty acid type	Notation*	Diet type (mg/g)		
		L18:2/L18:3	L18:2/H18:3	H18:2/L18:3
Lauric acid	12:0	22.4	—	—
Myristic acid	14:0	13.1	0.4	—
Palmitic acid	16:0	19.1	16.2	17.9
Stearic acid	18:0	5.8	5.3	5.3
Oleic acid	18:1	22.4	23.8	23.3
Linoleic acid	18:2	23.4	27.8	45.6
α -Linolenic acid	18:3	13.0	26.5	11.9
Total PUFA	—	36.4	54.3	57.5

* The number to the left of the colon indicates the number of carbon atoms, the number to the right denotes the number of carbon-carbon double bonds (Gunstone, 1996).

Laboratory Hibernation Experiment

Two *S. lateralis* groups (N = 6 each) were maintained on different semisynthetic diets for 3 months; one was fed the H 18:2/L 18:3 diet while the other was given the L 18:2/H 18:3 diet. Both groups were then placed at 5° C in an incubator during October 2000 to induce hibernation. All food was withheld after a two-day adjustment period. All squirrels not displaying torpor during 10 days of fasting were removed from the incubator and fed their regular semisynthetic diets for three more weeks. These squirrels were then fasted inside the incubator again for another 10 days to induce hibernation, and any squirrel not displaying torpor was removed from the study. All hibernating squirrels were maintained at 5° C for four months, and body temperatures were continuously recorded using "Mini-Mitter" transmitters surgically implanted into the abdomen of each squirrel.

Diet Preference Trials

Two consecutive diet selection experiments were conducted during October 2001 with 16 captive adult *S. lateralis*; both involved the L 18:2/L 18:3 and L18:2/H 18:3 diets. Each squirrel was given 120 g of each diet simultaneously at the onset of the first experiment. All remaining pellets were collected after six days. All squirrels were then placed in the second experiment by presenting each with 80 g of both diet types. All remaining pellets were collected after four days. The remaining pellets recovered at the end of each experiment were sorted by diet type (color), dried, and weighed. The amount of each diet consumed was calculated as the difference between the amount of dry matter initially presented and that remaining.

Overwinter Survival Study

Fifteen free-ranging *S. lateralis* were examined in the Barcroft area (37° 35.013' N, 118° 14.208' W, elevation = 3776 m) of the White Mountains of California. Torpor patterns and over-winter survival were monitored during the September–June periods of 2000–2001 and 2001–2002 using radio collars placed on each individual that transmitted pulse rates corresponding to skin temperatures. Blood plasma PUFA concentrations depend mostly on diet PUFA content during feeding (Gunstone, 1996). Blood samples were thus collected from each radio collared squirrel just prior to the onset of torpor (between 1 September and 5 October) and analyzed for plasma fatty acid content to provide a relative index of individual diet PUFA level.

Results

Laboratory Hibernation Experiment

Only three of the six squirrels fed the H 18:2/L 18:3 diet eventually displayed torpor, whereas a significantly greater proportion, six out of six, of those given the L 18:2/H 18:3 diet did ($FI = 41.3$, $P < 0.05$). All squirrels in the L 18:2/H 18:3 diet group continued to display regular torpor bouts throughout the four-month period at $T_a = 5^\circ \text{C}$, but two of the three squirrels from the H 18:2/L 18:3 group that initially entered torpor spontaneously stopped displaying regular torpor bouts during this same period. Hibernating members of both groups displayed statistically equivalent minimum body temperatures (Fig. 1A) and torpor bout lengths (Fig. 1B), however.

Diet Preference Trials

The mean (\pm SE) amount of the L 18:2/L 18:3 diet consumed during the first preference trial was $55.8 \pm 4.9\text{g}$ whereas that for the L 18:2/H 18:3 was $33.9 \pm 4.3\text{g}$ and significantly less (paired $t = -3.45$, $P = 0.004$). The mean (\pm SE) amount of the L 18:2/L 18:3 diet ingested during the second experiment was $43.9 \pm 4.9\text{g}$ and significantly more ($t = -2.86$, $P = 0.01$) than the amount of the L 18:2/H 18:3 diet consumed ($26.5 \pm 3.5\text{g}$) during the same period. The L 18:2/L 18:3 acid diet constituted a mean (\pm SE) proportion of the total food

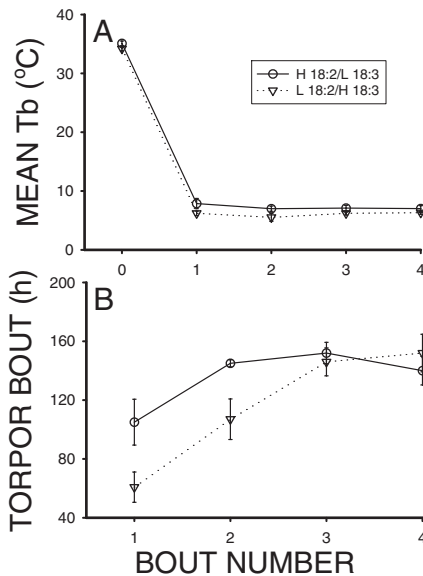


Fig. 1 (A). Minimum body temperatures recorded for each animal during torpor by *S. lateralis*. (B) Duration of torpor bouts (h) by *S. lateralis* in different diet groups. Symbols indicate means, vertical lines represent \pm SE.

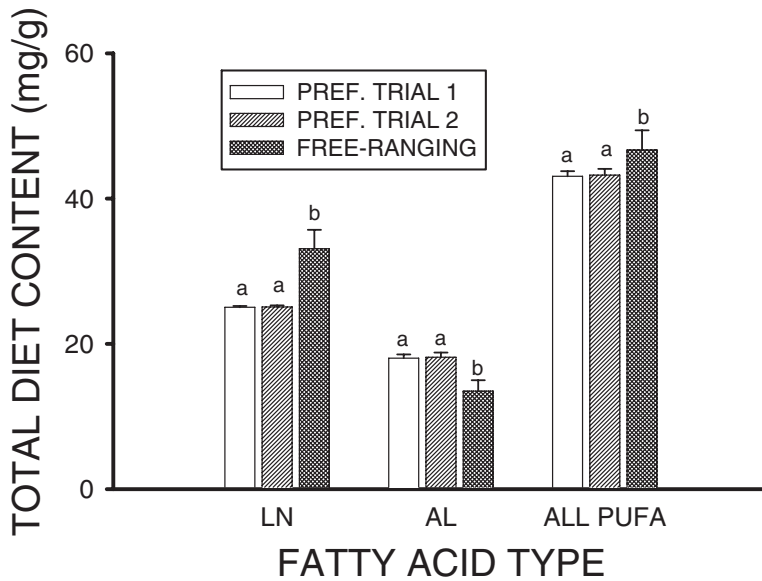


Fig. 2. Histograms indicating the mean (\pm SE) total intakes of linoleic (LN) and α -linolenic (AL) acids during the selection experiments. Data for free-ranging *S. lateralis* are from Frank et al. (1998). Means sharing a common lowercase letter are not significantly different at the $P < 0.05$ level.

ingested that was $62.8 \pm 3.8\%$ during the first experiment, whereas this fraction was $61.8 \pm 4.8\%$ during the second experiment and did not significantly differ between the two experiments ($t = 0.25$, $P = 0.81$). Total 18:2 intakes were calculated as the fraction of the combined amounts of each diet consumed during an experiment that was 18:2, and total 18:3 intakes were calculated in a likewise fashion. The two preference experiments did not significantly differ in total 18:2 ($t = 0.25$, $P = 0.41$), total 18:3 ($t = -0.25$, $P = 0.81$), or overall PUFA ($t = -0.26$, $P = 0.81$) intakes (Fig. 2). The total 18:2 intakes observed during both laboratory experiments were significantly lower (Fig. 2) than those observed for the natural diets of free-ranging *S. lateralis* by Frank et al. (1998) (Student's $t = 47.8$, $P < 0.001$, and $t = 38.3$, $P = 0.002$, respectively), whereas both laboratory total 18:3 intakes observed (Fig. 2) were significantly greater ($t = 8.7$, $P < 0.001$, and $t = 7.3$, $P < 0.001$, respectively). The natural diets of free-ranging *S. lateralis* thus had significantly greater overall PUFA intake (Fig. 2) than those of the first ($t = -5.3$, $P < 0.001$) and second ($t = -4.1$, $P = 0.002$) laboratory selection experiments.

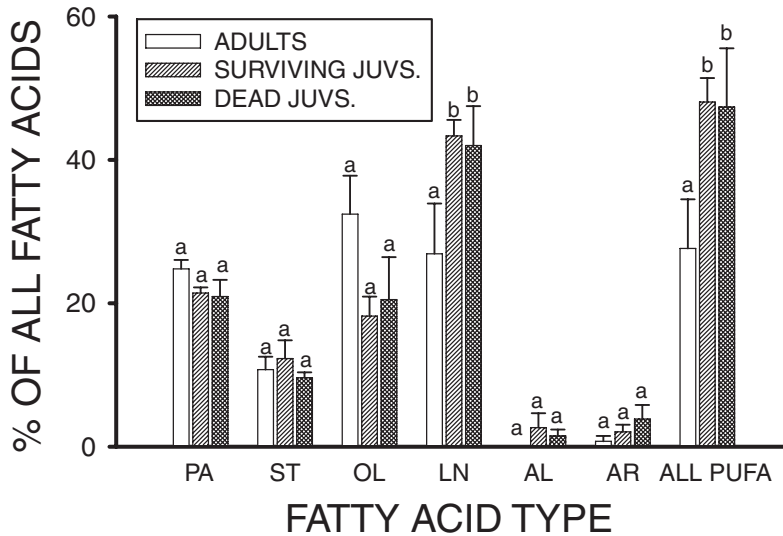


Fig. 3. Histograms indicating the mean (\pm SE) levels of palmitic (PA), stearic (ST), oleic (OL), linoleic (LN), α -linolenic (AL), and arachidonic (AR) acids found in the blood plasma of free-ranging adult and juvenile *S. lateralis*. Means sharing a common lowercase letter are not significantly different at the $P < 0.05$ level.

Over-Winter Survival Study

All five of the radio-collared adult *S. lateralis* survived the winter, whereas only 7 of the 10 radio-collared juvenile *S. lateralis* survived during this same period. The fall blood plasma of adults, juvenile survivors, and juveniles that subsequently died did not significantly differ in palmitic (GLM ANOVA $F = 2.80$, $P = 0.10$) stearic ($F = 0.30$, $P = 0.75$), oleic ($F = 3.40$, $P = 0.07$), α -linolenic ($F = 3.77$, $P = 0.51$) or arachidonic ($F = 1.42$, $P = 0.28$) acid contents (Fig. 3). Both juvenile groups had significantly greater overall plasma PUFA levels, however ($F = 4.53$, $P = 0.03$) than the adults (Fig. 3), due to correspondingly greater linoleic acid ($F = 3.77$, $P = 0.05$) levels (Fig. 3).

Discussion

Squirrels fed the L 18:2/H 18:3 diet had a greater propensity to both enter and remain in torpor than those given the H 18:2/L 18:3 diet, even though these two diets had virtually identical total PUFA contents (Table 1). These findings support our hypothesis that moderate diet α -linolenic acid levels enhance mam-

malian torpor. They are also in contrast to those of Hill and Florant (2000) in which their 4.5% linseed oil diet actually inhibited torpor, even though the α -linolenic acid content was only 20.6 mg/g. Hill and Florant (2000) were surprised by their finding and speculated that their results were due to compounds in the linseed oil diet other than α -linolenic acid content. Our study supports their supposition. Diet α -linolenic acid content was not maximized by *S. lateralis* during the laboratory selection experiments but was instead maintained between 18.0 and 18.2 mg/g, on average. The susceptibility of α -linolenic acid to peroxidation is far greater than that of linoleic acid (Gunstone, 1996). Interpreting the diet selection experiments in conjunction with this fact suggests that *S. lateralis* maintains a diet α -linolenic acid content of 18.0–18.2 mg/g to both maximize torpor propensity while minimizing the rate of tissue lipid peroxidation during torpor. The blood plasma of juvenile *S. lateralis* contained nearly twice the level of linoleic acid as that of adults collected during same period, indicating that they have a correspondingly higher diet linoleic acid content during the fall. Exceedingly high levels of linoleic acid in the fall diet inhibits torpor (Frank, 2002), thus the greater over-winter mortality of juveniles may be due in part to their fall diet selection. Further investigation of this system may therefore provide valuable insights into the ecological/nutritional constraints on mammalian torpor.

Acknowledgments

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Heat Transfer in Humans: Lessons from Large Hibernators

DENNIS GRAHN AND H. CRAIG HELLER

Department of Biological Sciences, Stanford University, Stanford, CA 94305

Abstract. Unique heat exchange vascular structures—that underlie nonhairy skin surfaces of most mammals (including humans)—enable direct thermal access to the body core. The combined application of subatmospheric pressure and a thermal load (either warm or cold) were used to enhance heat transfer through these radiator structures and directly deliver heat to, or extract heat from, the body core. Using this technique, normothermia was rapidly restored in cold-stressed SCUBA divers, and the temperature balance of heat-stressed recreational athletes was affected during exercise and post-exercise recovery.

Introduction

Vasomotor tone is a primary thermoregulatory effector mechanism and vasoconstriction is the initial response to a cold challenge (Wyss et al., 1974). Both central and peripheral inputs, either alone or together, can elicit a vasoconstriction response; a decrease in core temperature can elicit a vasoconstriction response as can an abrupt decrease in skin temperature (Hales et al., 1985, Frank et al., 1999). These vasomotor responses, while appropriate for protecting against external insults, also limit the transfer of a thermal load from the skin to critical core regions of heat- and cold-stressed individuals. Cold-stressed individuals are, by nature, vasoconstricted. Thus, the application of a warm stimulus to the general skin surface, while effective for increasing skin temperature, has little immediate effect on the temperature of the body core. Afterdrop—a continued decline in core temperatures of hypothermic individuals after removal from the cold and the initiation of superficial heat application—is a well-documented

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

phenomenon (see Giesbrecht and Bristow, 1998). Similarly, the application of surface cooling to a heat-stressed individual can elicit a vasoconstrictive response (Crawshaw et al., 1975; Pergola et al., 1996). If the cold application elicits a vasoconstrictive response, the treatment will have little or no immediate effect on core temperature and may even activate inappropriate thermogenic mechanisms (Kashmeery, 2000). When treating heat- or cold-stressed individuals, the challenge is to deliver an appropriate thermal load to the body core despite the physiological defense mechanisms. The study of large hibernating mammals has led to a unique insight as to how thermoregulatory mechanisms can be manipulated to enable direct access to the thermal core of humans.

Most mammals maintain relatively constant internal temperatures despite fluctuations in environmental conditions and internal heat production. To achieve this end, an animal must be able to: (1) protect the internal milieu from external thermal challenges, and (2) dissipate excess internally produced heat. Further, this must be achieved without exhausting the limiting resources of metabolic fuel in the cold and body water content in the heat.

In the subarctic regions, annual temperature and nutrient resource cycles are extreme. Winters are cold and food is scarce; summers are warm and food is abundant. Animals that do not migrate must endure these extreme annual cycles. Black bears, as seasonal hibernators, are inactive and hypometabolic in the winters and, thus, metabolic heat production is lowest during the coldest environmental conditions (Nelson et al., 1973; Barnes et al., 1999). Despite the reduced metabolic activity and behavioral quiescence, black bears maintain relatively high core temperatures throughout the winter (Barnes et al., 1999). The relatively high core temperatures are enabled largely by a thick coat of fur and a thick layer of subcutaneous fat that protects the internal tissues from the external cold. Heat loss is further minimized by passing respiratory gasses through nasal turbinates—a dense matrix of thin-wall calcified structures in the snout—which form a network of small-diameter channels. The ebb and flow of air through the turbinates create a heat trap to reduce respiratory heat loss.

In the spring when the black bear emerges from hibernation, the demands on the thermoregulatory system are reversed, transitioning from protecting against the cold to preventing overheating. The insulation that enables a high core temperature throughout the cold winters must also limit heat dissipation capacity during the warm summers. Yet, despite a four-to-six-fold increase in basal metabolic rate and increased motor activity, bears neither shed their fur nor substantially alter their subcutaneous insulation. Therefore, these animals must have a

means for dissipating excess internally produced heat despite the thick layers of insulation.

Limited regions of the bears' body surfaces lack external insulation: the pads of the feet, the tip of the nose, and the tongue. Underlying these hairless surfaces are unique vascular structures capable of delivering large volumes of blood directly to the subcutaneous space. In these animals, thermoregulatory vasomotor responses are confined to these heat exchange vascular structures underlying the nonhairy surfaces and, thus, these surfaces enable heat loss despite the insulation (Tøien et al., 1999). Similar unique vascular structures underlie limited regions of the bare skin surfaces of most mammals (e.g., flippers and flukes of marine mammals, the tongues and foot pads of canines, the tails of rats, the ears of rabbits and elephants) and serve to dissipate excess internal heat.

The surface of the human body is covered by two types of skin: glabrous (nonhairy) and nonglabrous (hairy). The glabrous skin regions are characterized not only by an absence of hair follicles but also by a unique set of subcutaneous vascular structures (arteriovenous anastomoses and venous plexuses) which enable large volumes of circulating blood to flow through the subcutaneous space (Greenfield, 1963). The glabrous skin surfaces are restricted to the palms of the hands, the soles of the feet, the ears, and limited regions of the face. These structures provide a pathway for blood to flow directly from the heart (via arterial outflow) to the body surface and back to the heart (via venous return). The venous plexuses are arranged to allow sustained residence time of the circulating blood directly under the exposed body surfaces. Sphincter muscles in the arteriovenous anastomoses control the blood flow through the venous plexuses (Bergersen et al., 1997). The absence of external insulation and the presence of the unique vascular structures underlying glabrous skin surfaces create radiator-like structures. Thus, while a thermal load applied to the general skin surface is mostly absorbed by local skin, muscle, and bone, a thermal load applied to these specialized structures is mostly absorbed by the blood that is returning to the body core (House et al., 1997; Vanggaard et al., 1999).

The limiting factor for heat transfer through these heat exchange vascular structures is vasomotor tone; vasoconstriction shuts down heat transfer (Berner et al., 1999; Johnson et al., 2002). We reasoned that if blood flow through the heat exchange vascular structures could be independently controlled, it should be possible to directly manipulate core body temperatures regardless of vasomotor drive. A local pressure differential provided a means for mechanically drawing blood into the distal portion of an appendage. A hand was placed inside a

rigid airtight chamber with a flexible seal around the wrist and a slight subatmospheric pressure (-40 mm Hg) was applied inside the chamber. Since the only expandable spaces inside the rigid chamber were the vascular structures, this minor pressure differential drew blood into the vascular structures and maximized the resident volume of blood inside the encased appendage. A thermal load, delivered by water circulating through a closed circulating system, was then applied to the skin surface overlying the distended heat exchange vascular structures. An initial clinical study established that the method was effective for rewarming (and eliminating associated tremor activity) surgical patients during post-anesthetic recovery (Grahn et al., 1998). To determine whether these results were unique to the peri-anesthetic setting, we assessed the effects of applying this thermal manipulation technique to cold- and heat-stressed, but otherwise healthy subjects.

Materials and Methods

General Methods

These studies were conducted under protocols approved by a Stanford University administrative panel on human subjects in medical research. Informed consent was obtained from all subjects prior to participation and each subject was assigned an alphanumeric identifier, which was used thereafter in accordance with HIPAA guidelines. Esophageal and/or tympanic temperatures (T_{es} and T_{ty}) were monitored using commercially available thermocouple probes (Mon-a-therm, Mallinckrodt Medical, Inc.) and the data collected on portable dataloggers (OM-3000, Omega Engineering, Inc.). All data was transferred to a computer for subsequent analysis. Data analysis entailed plotting the raw data, grouping the data sets, and calculating mean and SEM values for grouped data sets.

Cold Stress

Subject population and environment: Numerous SCUBA diving schools in Northern California meet at the Coast Guard Pier in Monterey, California, each weekend to teach introductory diving. The first time SCUBA divers are generally poorly equipped, naive to the effects of cold water immersion, and need to make multiple dives to fulfill their certification requirements. These studies were conducted on three weekends in January and February when the water and air temperatures in Monterey Bay both averaged 12° C. Informed consent was obtained from a large group of potential subjects on the morning of their dives before they entered the water. Divers were in the water for 40–60 minutes

per dive. Upon their return to the land, a substantial portion of the previously consented novice divers were eager to participate in a heat-related study. Results from 21 subjects are reported here.

Procedures: Upon emerging from the water, the subjects were equipped with a T_{ty} probe. The warming device used here was similar to the warming device that had been used in the previous clinical trials. The first 5 min. of temperature monitoring established baseline temperature profiles, after which the subjects sat for 20 minutes with the warming device applied to one hand. The temperature of the water flowing through the device separated the experimental from control treatments ($45\text{--}46^\circ\text{C}$ for the experimental treatments, $25\text{--}30^\circ\text{C}$ for the controls). In the control trials, the temperature of the water circulating through the device was increased to $45\text{--}46^\circ\text{C}$ after the 20 minutes of treatment. The subjects remained clad in their wet suits during treatments. Shivering and vasomotor tone were also recorded at three-minute intervals. Shivering was assessed on a binary scale; visual tremors were either present or absent. Vasomotor tone was based on nontreated hand temperature; if the hand was cold to the touch, the subject was vasoconstricted; if warm, the subject was vasodilated.

Heat Stress Induced by Exercise in a Hot Environment

Equipment and facilities: All exercise trials were conducted either on a stationary treadmills or a cycle ergometer in a hot room ($34 \pm 1.0^\circ\text{C}$, RH 30–50%). This study was conducted in a temperature-controlled environmental chamber located in the Department of Biological Sciences, Stanford University. The cooling device was similar to the rewarming device described above except for the temperature of the circulating water.

Procedures: The subjects were equipped with T_{es} thermocouples. The subjects then entered the hot room and after 5 minutes of rest began exercising. Each subject used the same exercise equipment in all of his or her trials and individual work loads (self-selected by the subjects) remained constant throughout the trials. The exercise stop-points were either subjective exhaustion or a T_{es} of 39.0°C . T_{es} was monitored throughout exercise and 30 min of post-exercise recovery. Individual trials were separated by a minimum of three days. For cooling the temperature of the circulating water was maintained at $22 \pm 0.5^\circ\text{C}$ and the pressure inside the device was maintained at $-40 \pm 5\text{ mm Hg}$ (-20 inches of water). For the control trials the device was applied to the hand but neither the vacuum nor water pump was activated.

The effect of hand cooling on T_{es} . Five fit recreational male athletes (ages 24–45)—clad in light shirts, shorts, and shoes—participated in two exercise trials each; one with and one without cooling through the hand during both exercise and recovery. The subjects remained in the hot environment during recovery.

The effect of ambient temperature and insulation on T_{es} during hand cooling. Eight subjects (five male and three female, ages 22–47) participated in four trials each. These subjects were clad in plastic rain pants and jackets and thick cotton, hooded sweat suits during exercise. In two of the trials the subjects remained in the hot environment and clad in the insulation layers during the post-exercise recovery. In the other two trials, the subjects immediately moved to a 17° C room and shed the insulating layers upon termination of exercise. Under both post-exercise recovery conditions the subjects received cooling and control treatments.

Results

Cold Stress

All subjects were subjectively cold, vasoconstricted, and shivering at the start of the trials. Pretreatment T_{ty} s ranged from 33 to 35° C. Control subjects were warmed after 20 min of sham treatment (Fig. 1). T_{ty} of all subjects treated with the warming device reached 36° C within 15 minutes. There was no appreciable change in T_{ty} of the control-treated subjects over the same time period (Fig. 2). When heat was delivered through the hand, shivering and vasoconstriction waned as T_{ty} increased. Shivering and vasoconstriction persisted in the control-treated subjects until after warming had been initiated.

Heat Stress

Heat extraction decreased the rate of T_{es} rise during exercise and increased the rate of T_{es} decline during recovery (Fig. 3). Changes in insulation and ambient conditions, while affecting the overall patterns of T_{es} during recovery, had little influence of the effect of heat extraction (Fig. 4). For instance, the rate of T_{es} decline was lower when the subjects remained fully insulated in the hot room than when the subjects removed their insulating clothing and moved to a cooler environment. However, under both conditions, the rate of T_{es} decline increased with cooling.

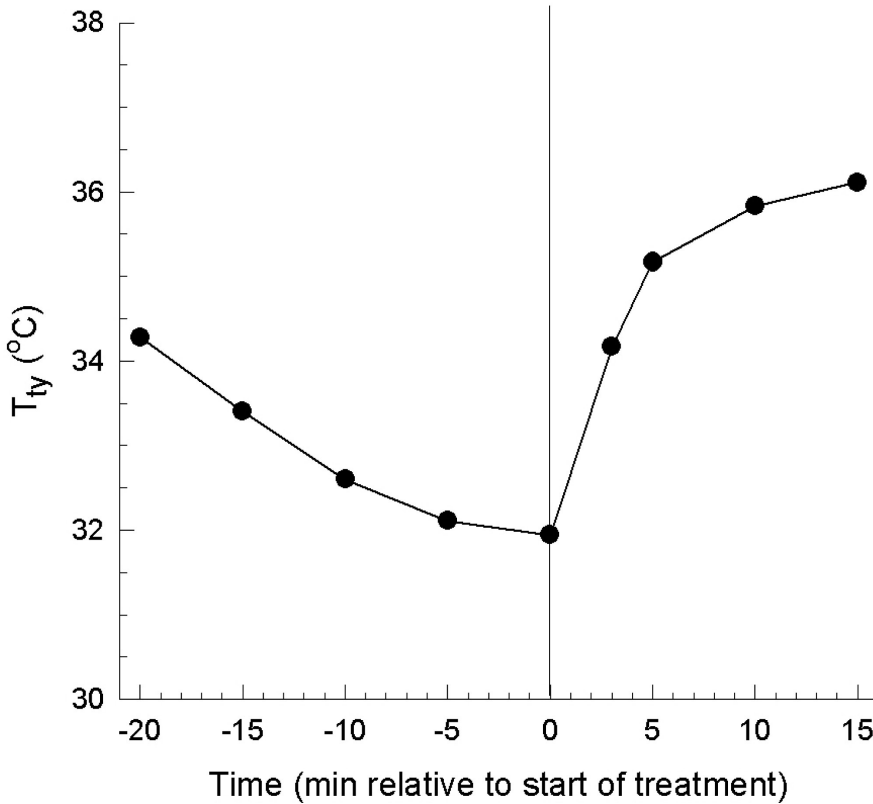


Fig. 1. Tympanic membrane temperature (T_{ty}) of a SCUBA diver during recovery from a dive. The subject was equipped with a tympanic membrane thermocouple probe upon emerging from the water. The subject sat in a thermoneutral environment (approximately 22° C) with one hand in the warming device. After 20 min of treatment the temperature of the circulating water was increased from 25° C to 45° C. Note the afterdrop in core temperature prior to the commencement of the warming. The afterdrop phenomenon—an initial decrease in core temperature during recovery from cold water exposure—is a common occurrence and has been well documented (see Giesbrecht and Bristow, 1998).

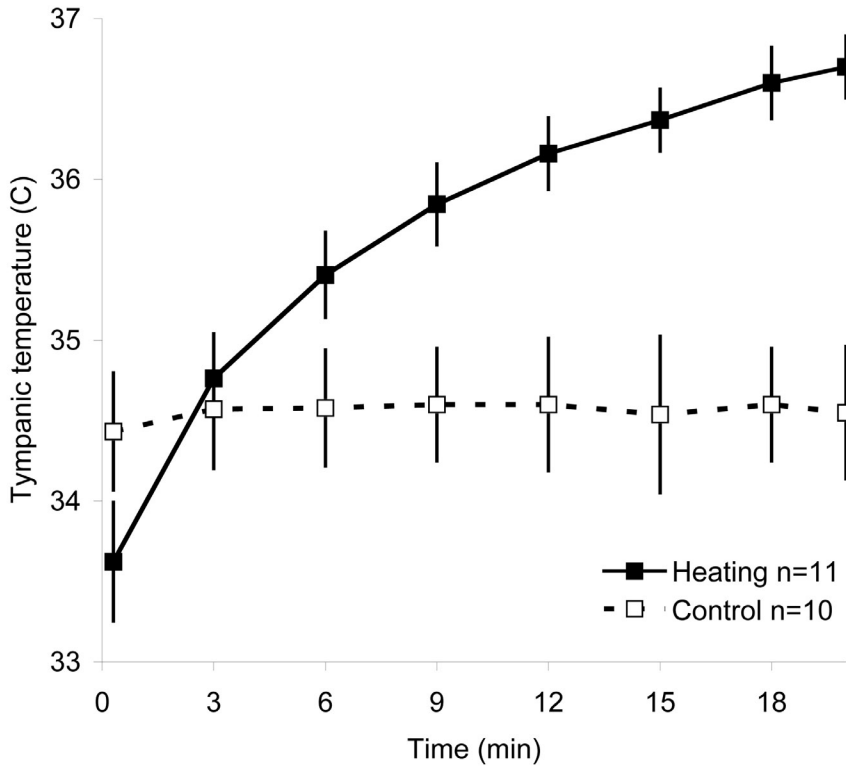


Fig. 2. T_{ty} during recovery after SCUBA dives. Closed symbols: warming by delivery of heat through the palm of one hand. Open symbols: control treatment. The temperature of the water flowing through the device separated the experimental from control treatments ($45\text{--}46^\circ\text{C}$ for the experimental treatments, $25\text{--}30^\circ\text{C}$ for the controls). The subjects remained clad in their wetsuits during treatments. Shivering was assessed on a binary scale; visual tremors were either present or absent. Shivering persisted through the recovery in the control subjects, but subsided in the experimental subjects within 10 minutes of treatment.

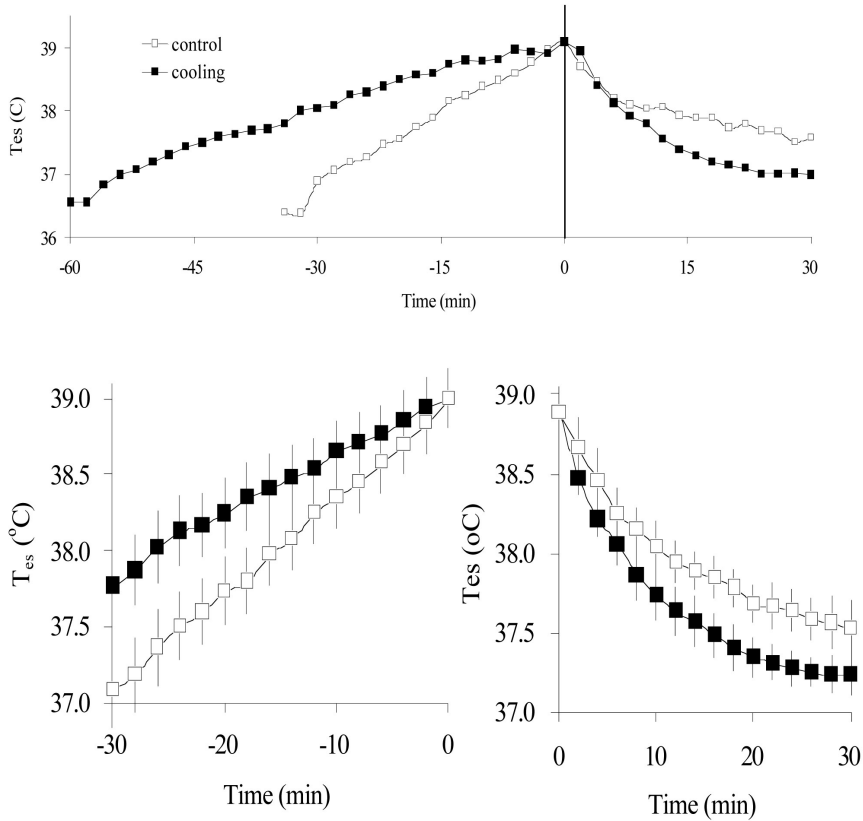


Fig. 3. Esophageal temperature (T_{es}) during exercise and recovery in a hot room. The rate of T_{es} rise during exercise was decreased with cooling, which enabled longer duration exercise bouts. Closed symbols: cooling through one hand. Open symbols: control treatment. Top panel: an example of one subject. Bottom panel: Group results (mean + SEM, $n = 5$) of T_{es} during the last 30 minutes of exercise (left) and post-exercise recovery (right).

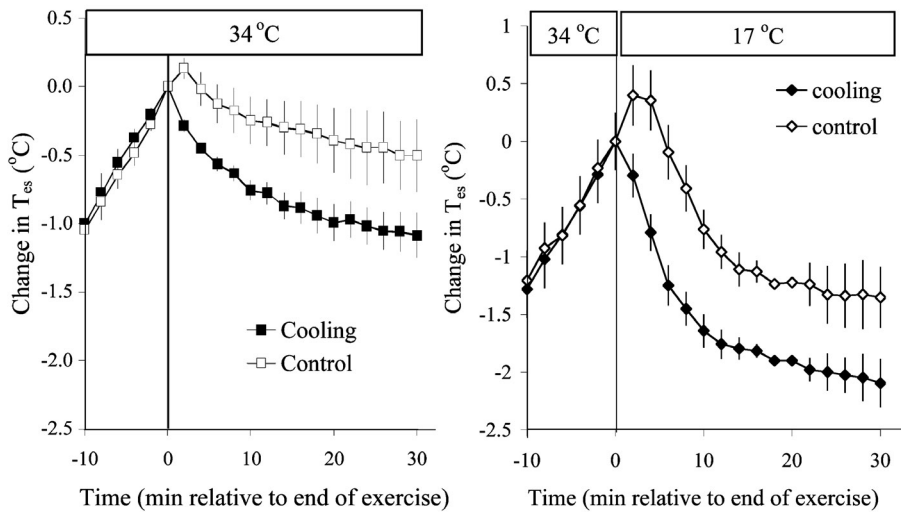


Fig. 4. The effect of insulating clothing and ambient temperature on heat extraction during post-exercise recovery. Left panel: subjects clad in insulation recovering in a hot room. Right panel: uninsulated subjects recovering in a cool room. Closed symbols: cooling through one hand. Open symbols: control treatment. The rate of overall cooling was affected by the insulation and ambient conditions. The subjects cooled more rapidly in the cool room without insulation. However, under both conditions cooling through one hand reduced T_{es} . The rate of T_{es} decline of the insulated subjects cooled during recovering in the hot room approximated the rate of T_{es} decline of the uninsulated subjects recovering in the cool room without treatment.

Discussion

These results demonstrate that the thermal core of both heat- and cold-stressed humans can be accessed through specific radiator structures underlying the non-hairy skin surfaces. There are three radiator regions in the human body available for exploitation—the soles of the feet and the palms of the hands, and nonhairy regions of the head. Only a single hand was treated in these studies. As more sophisticated interface components are developed (i.e., ones that take full advantage of the heat-dissipation anatomy and can be used on both the hands and feet), it will be possible to deliver a greater thermal load to the body core. The ability to deliver a substantial thermal load directly to the body core should dra-

matically affect survival times in extreme hot and cold climates. The ability to directly manipulate core temperature also has immediate medical applications.

A critical factor for utilizing the radiator structures for manipulating core temperature is local temperature. The pressure differential draws blood into the venous plexuses, thereby affecting the volume of the blood in the structures, but the pressure differential by itself has no effect on blood flow through the system. If the gating mechanisms in the AVAs are not open, the temperature manipulation will have no effect on core temperature since the warmed or cooled blood will not return to the core. Interface temperature is critical for enabling blood flow through the AVAs. In the heat-stressed trials water perfusion temperature was kept above 20° C to prevent local vasoconstriction. For cold-stressed individuals, local temperatures of 45° C were necessary to release the centrally driven vasoconstriction. That the pressure differential alone does not override central vasomotor control provides a physiological autoregulatory feature to prevent overshooting thermoneutral core temperatures during treatment. If during recovery from heat stress the vasoconstriction threshold is reached, blood flow through the hand will be shut off. If, during treatment for cold stress, a core temperature threshold for vasodilation is achieved, the remaining heat exchange vascular structures will dilate and heat being delivered through the treated appendage will be dissipated through the other AVAs and venous plexuses.

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Factors Influencing the Timing of Dormancy in the Pocket Mouse, *Perognathus longimembris*

ALAN R. FRENCH

Penn State University, Department of Biology, Lehman, Pennsylvania, USA, and Binghamton University, Department of Biology, Binghamton, New York, USA

Abstract. Seed availability was controlled in pocket mice, *Perognathus longimembris*, kept in artificial burrow systems at 5° C, to determine if the timing of their phases of surface activity and underground dormancy were responsive to input of energy. Mice unable to accumulate a large cache of seeds did not initiate dormancy, and some mice may have terminated dormancy when their food reserves became low. However, when food was not limiting, the behavioral transitions appeared to occur spontaneously. Mice provided with a cache of seeds ceased foraging even though food was constantly available on the surface, and later most terminated dormancy with abundant food uneaten. The timing of these events was not related to the amount of food stored. The rate of food consumption was directly related to the amount of food stored, which indicated that, on average, mice with more food spent less time in torpor. In the absence of environmental cues, the duration of dormancy varied greatly. Fifty-four percent of females and 11% of males remained dormant over a year, and some stayed underground for as long as 3.5 years. Emergence was stimulated in some mice by raising the temperature, and similar thermal cues are probably important for synchronizing emergence in nature.

Introduction

Like other hibernators, *Perognathus longimembris* annually undergo profound changes in behavior. These small (~8 g) pocket mice are active above ground in the spring and summer, but then cease foraging and remain underground,

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subsisting on stored seeds, until spring of the next year (Grinnell and Swarth, 1913; Kenagy, 1973). Captive mice also alternate intervals of surface activity and dormancy when kept in burrow systems under constant temperature and photoperiod, but the durations of successive intervals vary greatly (French, 1977). It is possible that, in the absence of other environmental time cues, mice forage as long as necessary to accumulate sufficient caches of seeds, and then remain dormant until those energy reserves have been depleted. In this study, the rate pocket mice harvested seeds and the amount they had stored at the start of dormancy were controlled to explore the possibility that the animals' behavioral patterns are responsive to energetic conditions.

Methods

Mice were captured in mid August in the Mohave Desert near Pearblossom, Los Angeles County, California, and shipped by air to New York. They were initially housed individually in plastic boxes containing a layer of fine grain sand and placed in a 22° C room with a 12 hours of light:12 hours of dark photoperiod. The mice, which do not drink water, were fed *ad libitum* a mixture of seeds containing approximately 70% millet, 20% rape, and 10% oats by weight. On 2 October, the animals were transferred to artificial burrow systems located within a 5±0.5° C room with the same photoperiod. Each burrow system consisted of a 48 x 30 x 30 cm wooden box with a screen lid and was divided into four vertical levels with plywood partitions. The upper level served as the ground surface. The three levels below the surface each supported a maze made of thin, 7 cm-tall boards, and a small ramp leading to a hole in the partition above. Each partition was supported by the boards below it and could be easily removed to facilitate excavation. A 3 cm layer of sand was placed in each underground level and on the surface, and cotton was provided for nest construction. Each mouse plugged the entrance hole to its burrow system with sand and emerged to forage only at night. The disappearance of seeds, or the disruption of powdered talc that was placed over the burrow entrance, indicated that the animal had emerged.

Forty-four animals were given a bulk ration of either 100 g of seeds (six mice), 200 g (14 mice), 400 g (18 mice), or 600 g (six mice) at the start of the experiment, and then an additional 1 g per day until they ceased foraging. The last 1g daily feeding remained on the surface throughout dormancy. Burrows were checked daily for the first year, and usually twice a week thereafter. Emergence was assumed to have occurred at the midpoint between the dates the mouse was found to have emerged and the preceding check. To investigate the

influence of rising burrow temperatures on emergence, an additional 28 animals were given 400g of seeds and allowed to hibernate in 5 °C for either two and one-half months (12 mice) or five months (16 mice). At those times, the burrow systems were moved into rooms kept at either 12 °C or 23 °C, and checked daily for five days to see if the mice resumed foraging. Three animals died during dormancy, one in each of the 100 g, 200 g, and 400 g feeding regimes. Finally, eight mice were not provided with an initial seed cache, but instead fed only 1 g nightly for five months. This daily ration was given even if the mice did not forage the preceding night.

Results

Cessation of Surface Activity

Only those mice that accumulated a large, underground cache of seeds entered dormancy. The eight animals fed only 1 g of seeds each night did not stop foraging during the five months their activity was monitored. One of these animals came above ground each night. The other seven emerged nightly during the first two-three months, but then foraged more sporadically, often skipping one or two nights between episodes of surface activity. In contrast, the 42 mice initially given 100-600 g of seeds harvested that bulk ration during the first day or two and then foraged for an additional 1 g of seeds per night for only a few days thereafter (Table 1). These mice spontaneously terminated surface activity after

Table 1. Averages of the durations of foraging and dormancy, food left after dormancy, and the rate of food consumption as a function of food supplied to pocket mice at 5° C. Sample sizes, in parentheses, follow values. Data on food consumption were unavailable for seven mice. Data from both sexes were combined when significant differences did not exist. Time in torpor was estimated from the equation in French (1976) derived from animals kept at 8° C, assuming 4.5 kcal/g of seeds consumed, a body mass of 8 g, and an assimilation efficiency of 94%.

food cached (g)	foraging duration (days±SD)	duration of dormancy		food uneaten (g±SD)	rate of food consumption (g/day±SD)	estimated % time torpid
		males (days±SD)	females (days±SD)			
100	7.6±4.1 (5)	280±54 (2)	532±127 (3)	22.2±21.9 (4)	0.190±0.076	94
200	10.0±9.0 (13)	308±207 (6)	505±325 (7)	86.6±46.3 (10)	0.357±0.123	83
400	14.5±9.6 (18)	201±15 (7)	584±397 (11)	282.3±56.2 (15)	0.443±0.255	78
600	7.8±4.6 (6)	435±380 (3)	551±249 (3)	372.7±139.9 (6)	0.554±0.284	71
Average	8.9±8.3	283±212	550±338			

an average of nine days, and the timing of their start of dormancy was not related to sex or the amount of food initially harvested (ANOVA; $p=0.800$ for sex, $p = 0.967$ for food).

Resumption of Surface Activity

Pocket mice kept under constant environmental conditions resumed surface activity after spending several months continuously underground. However, the duration of dormancy was quite variable, ranging from 4.5 months to over 3.5 years. A two-way analysis of variance showed a significant effect of sex on the duration of dormancy ($p = 0.042$), but no effect of food ration ($p = 0.938$) and no interaction between food and sex ($p = 0.798$). Over half (54.2%) of the females remained underground for over a year, whereas only 11.1% of the males did so (Fig. 1). The median and mean durations of dormancy were 208 days and 283 days respectively for males, and 355 days and 550 days respectively for females (Table 1). The values for females would have been even greater had I not prematurely terminated the experiments of two animals. The burrow systems of these two females were excavated 980 and 987 days after the mice began dormancy when I assumed, erroneously, that they must have died while underground.

It appears that most mice kept under constant environmental conditions emerged spontaneously, and not in response to an energetic emergency. Two animals given 100 g and two given 200 g emerged after consuming over 90% of their food reserves, but the majority resumed surface activity with substantial portions of their food cache uneaten (Table 1). There was a significant ($p < 0.0001$), positive relationship between the amount of seeds given and the amount remaining after dormancy (Fig. 2).

The use of torpor, as indicated by the rate of food consumption, was inversely related to the amount of food cached prior to dormancy (Table 1). Rate of food consumption was significantly ($p = 0.0099$) related to food given when tested with a linear regression model, but only 16.5% of the variance was explained. Most of the variability was found among the mice given abundant food. Animals with low rates of food consumption, and hence high use of torpor, were in all feeding regimes. However, many animals given 400 g and 600 g ate their seeds at sufficiently high rates to suggest that they employed torpor only infrequently.

Many, but not all, mice emerged in response to five-day elevations in temperature. An increase from 5° C to 23° C appeared to be more effective than an

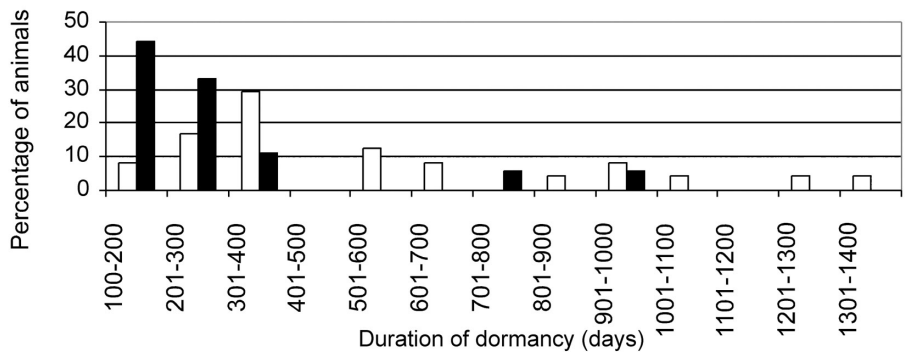


Fig 1. The proportions of male (dark bars) and female (light bars) pocket mice that remained underground for different durations of dormancy at 5° C.

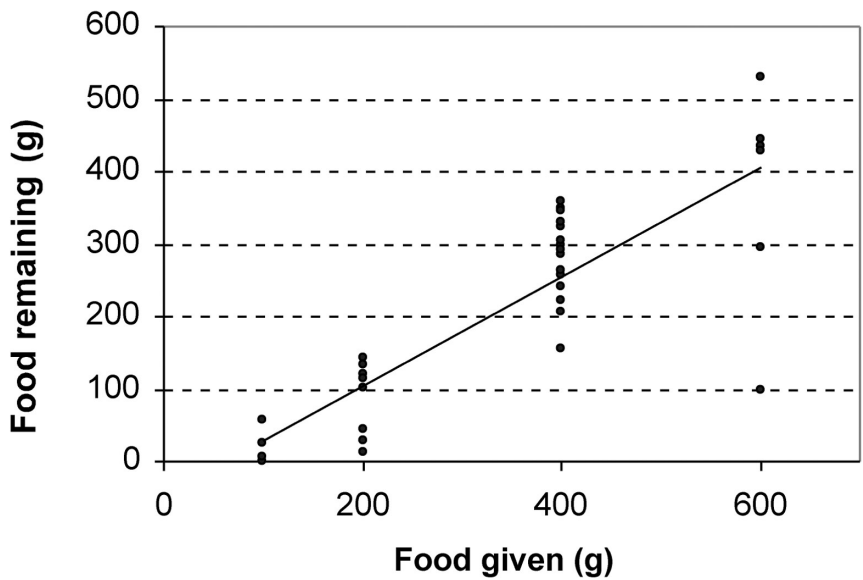


Fig. 2. The amount of food remaining at emergence as a function of the amount cached prior to dormancy in pocket mice at 5° C. Line is the least-squares regression of the data ($r^2 = 0.709$).

increase to 12° C, and the effectiveness of such temperature changes appeared to improve when given five months after the start of dormancy compared to only two and one-half months (Table 2). In fact, all mice that experienced 23° C after hibernating five months emerged. However, with such small sample sizes these differences were not statistically significant.

Table 2. Number of mice that did and did not emerge within five days of a rise in temperature after two and one-half and five months of dormancy.

sex	2.5 months				5 months			
	12° C		23° C		12° C		23° C	
	yes	no	yes	no	yes	no	yes	no
M	2	0	2	1	2	1	4	0
F	1	2	2	1	2	2	5	0

Discussion

P. longimembris kept in a constant environment made spontaneous transitions between phases of surface activity and dormancy when food was abundant. These behavioral changes are similar to, but much more variable than, the rhythms of torpor, body weight, and appetite seen in Sciurid rodents (Pengelley and Fisher, 1963; Heller and Poulson, 1970). The fact that some mice remained dormant for over three years demonstrates a profound flexibility unrivaled by other species of hibernators, and it underscores a dependency on environmental events to time behavioral changes.

In nature, pocket mice probably remain active just long enough to reproduce and gather a sufficient seed cache, both of which are influenced by environmental productivity. O'Farrell et al. (1975) found that in typical years, *P. parvus* reproduce and remain active for about 60 days. In unproductive years, mice do not breed and spend about 90 days foraging for the scarce seeds, whereas in highly productive years, individuals may have several litters and thus greatly delay their start of dormancy. The *P. longimembris* used in this study were non-reproductive and certainly ready for hibernation, because they entered dormancy almost immediately after they harvested their bulk rations. Only those animals unable to accumulate a seed store kept foraging. Obviously, 100 g of seeds is above the minimum the mice deem necessary for over-winter survival. Although the mice in this study were at 5° C, low temperatures are not necessary for the onset of dormancy. Adults stop foraging in mid summer when desert tempera-

tures are at their highest, and captive animals alternate phases of surface activity and dormancy when kept at 31° C (French, 1977).

The termination of dormancy in nature is correlated with the overturn of the winter temperature gradient in the soil (French, 1977). At this time of vertical uniformity, temperatures at the level of the animals' nests are just starting to rise. However, the soil surface is warmed daily, and now the mice no longer have to traverse through progressively colder layers of soil to reach it. During the spring and summer when the mice are active, they rest during the day in these warm upper layers (Kenagy, 1973). Certainly temperature changes can affect the behavior of *P. longimembris*. A uniform elevation in temperature to either 12 °C or 23° C stimulated emergence in 58% and 87% of the captive mice, respectively (Table 2). Although not statistically significant, the data suggest that a large rise in temperature is more effective than a smaller one, and the animals' sensitivity to temperature change may increase as dormancy progresses. Daily oscillations of temperature, like those a mouse would experience near the soil surface in nature, might be a more effective stimulus for emergence.

The direct relationship between food availability and the rate that food was consumed indicates that *P. longimembris* adjust their metabolism, presumably by the use of torpor, to energetic stress. This has been long known in species that enter daily torpor in response to immediate energetic emergencies (e.g. Tucker, 1966), but it has been documented also in *P. inornatus* (French, 1993) and the chipmunk, *Tamias striatus* (French, 2000) which are seasonal hibernators that store food. The matching of torpor to energy availability was imprecise, and in both species, animals with abundant food entered torpor more than necessary to survive the winter. Likewise, mice in this study must have been torpid and yet most had substantial food reserves when they emerged. A cache of seeds is not only important for over-winter survival, but it may be crucial during the succeeding active season especially in environments such as deserts whose productivity is so unpredictable.

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The Energetic State-dependency of Autumn Immergence in Eastern Chipmunks

MURRAY M. HUMPHRIES AND BRANDON RODGERS

Department of Natural Resource Sciences, Macdonald Campus, McGill University, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada

Abstract. If hibernators use accumulated energy reserves to avoid both torpor and predators, individuals with large autumn energy reserves should terminate above-ground activity sooner, but initiate torpor expression later, than individuals with small reserves. We evaluated this hypothesis in free-ranging eastern chipmunks (*Tamias striatus*) by experimentally supplementing burrow food hoards of some individuals, then comparing their autumn behavioral activity and torpor expression to controls. Food supplementation did not cause chipmunks to reduce activity prior to immergence, nor to immerge sooner. Instead all individuals, regardless of treatment, terminated above ground activity during a 2–3 week period of unseasonably cool temperatures in mid-October. However, food supplementation did significantly delay the initiation of deep torpor bouts ($T_{\text{transmitter}} < 20^{\circ}\text{C}$) following immergence. All individuals began expressing torpor within one or two weeks of terminating above-ground activity, but food supplemented chipmunks initiated deep torpor bouts on average 23 days later than controls. These results support the torpor avoidance hypothesis, but provide no evidence of energetic state-dependency in the termination of above-ground behavioral activity in autumn.

Introduction

Although torpor expression can be essential for surviving prolonged periods of energy shortage, hibernators may benefit from reduced torpor expression when surplus energy is available (French, 1988; Humphries et al., 2003a). This latter

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expectation is based on the existence of important physiological costs of torpor (e.g., immunosuppression (Prendergast et al., 2002), neuronal atrophy (Popov et al., 1992), oxidative stress (Carey et al., 2003), and inhibition of gametogenesis (Barnes et al., 1986)) that create a trade-off between the energy-saving benefits and somatic costs of torpor. Previous research on eastern chipmunks in the laboratory (French, 2000) and the wild (Humphries et al., 2003b), has demonstrated an inverse relationship between food availability and winter torpor expression. During each of two winters, Humphries et al. (2003b) found that chipmunks given large food hoard supplements in autumn spent less than half as much time in torpor during winter as individuals with non-supplemented hoards. Munro et al. (in prep.) recently repeated this result using continuous monitoring of torpor expression by free-ranging chipmunks, and have confirmed that food-supplemented individuals express much shallower and shorter torpor bouts than controls. The collective results of these studies offer clear support for the torpor avoidance hypothesis in chipmunks, but studies on other hibernating species are generally lacking (Humphries et al., 2003a).

The torpor avoidance hypothesis may be contradicted by the seasonal tendencies of several hibernating species to become inactive earlier in years of high resource availability than in years of low availability (O'Farrell et al., 1975; Michener, 1984). This might suggest that hibernators immerse as soon as sufficient reserves have been accumulated, rather than using available resources to delay hibernation as would be predicted by the torpor avoidance hypothesis (Humphries et al., 2003a). The solution to the apparent paradox of minimizing torpor during hibernation but maximizing the length of hibernation may involve hibernacula serving as a predator refuge as well as an energetic refuge. Most hibernators, including chipmunks, face much higher predation risks when above-ground than when inside their burrows (Humphries et al., 2003a). Behavioral ecological theory suggests occupation of predator refuges should vary in a state-dependent manner, with animals in better states (in this case with larger accumulated energy reserves) using refuges more than animals in poor states (Lima and Dill, 1990). Consistent with this prediction, eastern chipmunks provided with hoard supplements in early summer spend much less time out of their burrow during the remainder of the summer than do controls (Humphries and Kramer, unpublished data).

If hibernators seek to avoid both predators and torpor, individuals with large energy reserves in autumn should terminate above-ground activity sooner, but begin expressing torpor later, than individuals with smaller reserves. In this

way, hibernators with large reserves benefit from the energetic luxury of reduced above-ground activity as well as reduced torpor expression. Evaluation of this hypothesis requires that individual energy reserves can be experimentally manipulated in the field and that the initiation of torpor expression can be measured separately from the termination of above-ground activity. Because eastern chipmunks rely on stored food during hibernation and live solitarily in burrows, individual energy availability can be manipulated directly, without altering the general availability of food in the above-ground environment. We accomplish these manipulations by placing a food-filled box over the burrow of designated chipmunks, allowing the burrow owner to transfer a specified amount of food into their food hoard, then removing the box from the environment (Humphries et al., 2003b). This simple experimental approach permits the unique opportunity to isolate the importance of energetic state in the behavior and physiology of free-ranging animals (Houston and McNamara, 1999). In the present study, we use the approach to evaluate the prediction that chipmunks with large energy reserves should terminate above-ground activity sooner, but initiate torpor expression later, than individuals with smaller reserves.

Methods

Research was conducted in autumn 2003 at McGill's Morgan Arboretum in Ste-Anne-de-Bellevue, Quebec, Canada (45.3° N, 73.3° W) on free-ranging eastern chipmunks (*Tamias striatus*). Eastern chipmunks are a relatively small (90–100 g), sciurid rodent that inhabits eastern North American deciduous forests (Elliot, 1978). Chipmunks are food-storing hibernators, establishing a larder hoard of tree seed in their burrow without significant weight gain, prior to their 5–6 month hibernation period (Humphries et al., 2002). The timing of chipmunk autumn immergence differs substantially between years (Elliot, 1978; Kawamichi, 1996; Munro and Thomas, unpublished manuscript). Captive eastern chipmunks have been shown to express prolonged torpor bouts of up to seven days at body temperatures as low as 5° C (Wang and Hudson, 1971; Pivorun, 1976). Food is consumed from the burrow hoard during periodic arousals, and digestion and assimilation appear to continue following re-entry into torpor (Humphries et al., 2001). Except for females with dependent young, individuals occupy separate burrows consisting of a complex array of tunnels, nest chambers, and food-storage chambers (Elliot, 1978). Individuals are active above-ground during the day and occupy small (0.2–0.4 ha), extensively overlapping home ranges (Elliot, 1978; Getty, 1981). The principal food of eastern

chipmunks throughout much of their range is seeds produced by hardwood trees such as oak (*Quercus sp.*), American beech (*Fagus grandifolia*), and sugar maple (*Acer saccharum*; Elliot, 1978). Seeds from these species mature and fall to the ground in September and October, and as a result chipmunks are characterized by intense autumn hoarding activity prior to initiating hibernation (Humphries et al., 2002). The autumn-seeding hardwoods that form the principal food source of eastern chipmunks are regarded as masting species, because trees in a given population synchronously produce large seed crops every two to several years, and no or small seed crops in the intervening years (Kelly and Sork, 2002). Thus, eastern chipmunks must cope with extremely high annual variation in food availability. At our Morgan Arboretum study site in 2003, American beech and red oak both produced moderate mast crops (Humphries pers. obs.).

During the first week of September, 19 adult chipmunks (5 males, 14 females) were collared with temperature-sensitive radio transmitters (Model PD-2CT, Holohil Systems Ltd., Carp, ON, Canada; transmitter mass, 3 g; calibrated from 2° C–45° C), fitted snugly under the chin, following methodology described by Humphries et al. (2003b). Temperature-sensitive transmitters mounted next to the skin have been shown previously to provide a reliable index of core body temperature of inactive animals, with a usual accuracy of $\pm 2^\circ\text{C}$ (e.g., Körtner and Geiser 2000a). Radio collared chipmunks were randomly assigned to control (n=11) and food supplement (n=8) treatments, balancing for gender and burrow location. Individuals assigned to the food supplement treatment were provided the opportunity to transfer approximately 1,600 g (31,500 kJ) of sunflower seeds to their burrow food hoard during a 1-2 week period (see Humphries et al., 2003b for detailed description of method). All 8 of the supplemented chipmunks completed transferring the 1,600 g of seed into their burrow hoards between September 21–29, after which there was no additional supplemental food provided at the site.

Following completion of food supplements, we used radio telemetry to monitor the above-ground activity and initiation of torpor expression by supplemented and control chipmunks. Twice daily between Oct 3 to Nov 10 chipmunks were located with handheld telemetry, and scored as either in or out of the burrow. Based on these visits, we determined the last day each individual was observed above-ground. Beginning in mid-late October and continuing until mid-December, transmitter temperatures of 11 chipmunks (5 controls, 6 food supplements) were continuously monitored using two data-logging receivers

(Lotek SRX 400-W21 equipped with antenna switching firmware) and arrays of up to 4, 6-element antennas. The transmitter temperature of each chipmunk was recorded approximately once every 3 min to allow precise determination of torpor entries and arousals. Based on these measurements, we calculated the maximum torpor bout duration and minimum torpor baseline $T_{\text{transmitter}}$ for each individual. We also present the first date when torpor baseline $T_{\text{transmitter}}$ first dropped below 20°C, which was the highest minimum torpor baseline $T_{\text{transmitter}}$ expressed by any one individual in this study. Due to small sample sizes and occasional departures from normality, non-parametric Kruskal-Wallis tests are used to evaluate the significance of treatment effects. Sample size of the two groups (controls and food supplements, respectively) is indicated as subscripts of the Mann-Whitney U test statistic.

Results

All study chipmunks were last observed above-ground between October 7 and October 19, and the timing of disappearance was identical for food supplemented and control individuals (controls: Oct 10 ± 2 days, food supplements: Oct 10 ± 2 days, $U_{11,8} = 44$, $p = 0.99$; Fig. 1). Prior to immergence, food supplemented and control chipmunks also spent similar amounts of time outside their burrow (controls: $17 \pm 5\%$, food supplements: $19 \pm 7\%$, $U_{11,8} = 35$, $p = 0.45$). Consistent with the pattern of immergence observed at a nearby study site in 1997 (Humphries et al., 2001), disappearance of most individuals coincided with two occurrences of unseasonably cool air temperatures (Fig. 2).

All control and food supplemented individuals initiated torpor expression within a week or two of terminating above-ground activity (Fig. 3). However, the initiation of deep torpor bouts ($T_{\text{transmitter}} < 20^\circ\text{C}$) differed substantially between treatments, with controls expressing their first deep torpor bout on average 23 days earlier than food supplemented chipmunks (controls: Nov 6 ± 6 days, food supplements: Nov 29 ± 4 days, $U_{5,6} = 3.0$, $p = 0.028$; Fig. 4a). By mid-December, controls continued to be characterized by longer maximum torpor bout lengths (controls: 2.5 ± 0.6 days, food supplements: 1.2 ± 0.3 days, $U_{5,6} = 24.0$, $p = 0.1$) and a non-significant trend toward lower minimum torpor temperatures (controls: $13.5 \pm 1.9^\circ\text{C}$, food supplements: $18.7 \pm 0.7^\circ\text{C}$, $U_{5,6} = 4.0$, $p = 0.04$) than food supplemented chipmunks (Fig. 4b, c).

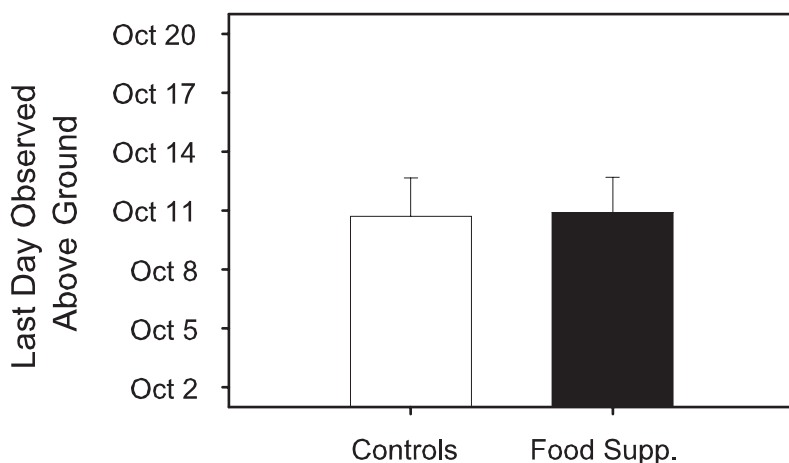


Fig. 1. Effect of energy reserves on the timing of autumn immergence by eastern chipmunks in 2003. Food supplemented individuals ($n=8$) had their burrow boards supplemented with 1,600 g of sunflower seeds in September. Above-ground activity by these animals and 11 controls was then monitored from October to mid-November using radio telemetry. Means are presented ± 1 standard error.

Discussion

The results presented here do not support the prediction that hibernators with large accumulated energy reserves terminate above-ground activity sooner than hibernators with smaller reserves, but do demonstrate that large reserves delay the initiation of deep torpor expression. Correlation evidence for the influence of energy availability on the timing of autumn immergence is mixed, with some studies reporting advancement of immergence under energetically favorable circumstances (e.g., O'Farrell et al., 1975) and others reporting a delay (e.g., Schooley et al., 1994). This is not surprising because abundant resources in the environment may have two confounding influences on state-dependent foragers; on the one hand, they may encourage additional foraging effort by enhancing the energetic benefits of foraging, but on the other hand, they may discourage additional foraging by facilitating the rapid accumulation of large reserves. Accordingly, natural or experimental increases in environmental resource availability may have only weak or temporally-contradictory effects on foraging activity. Individual manipulation of energetic state without altering the general availability of food in the environment provides a better test of state-dependent

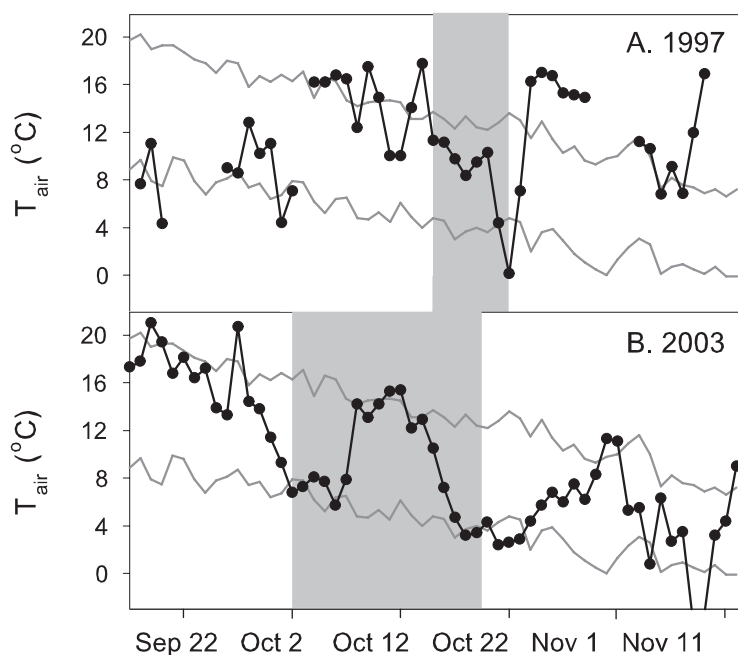


Fig. 2. Relationship between ambient temperature and the timing of autumn immersion by chipmunks in 1997 (A) and 2003 (B). Gray lines indicate normal daily highs and lows for the region, while dark lines and dots indicate observed average daily temperatures in the year of study. The broad grey lines indicate the range of dates different chipmunks were last observed above-ground. Fig. 2A is redrawn from Humphries et al. (2002) and is based on data collected at a different, but nearby, study site.

immersion, but despite using this approach, we detected no effect of energetic state on the timing of immersion. Thus, evidence available to date suggests that external influences such as weather (Lehmer et al., 2003), environmental food availability (Kawamichi, 1996), or photoperiod (Körtner and Geiser, 2000b) are more important than individual energetic state in dictating immersion behavior in eastern chipmunks. However, we would like to repeat this study in additional years of varying environmental conditions, with more intensive sampling of pre-immigrant activity and different combinations of food types and timing of food supplementation, before we are confident in rejecting a role of energetic state in the timing of autumn immersion by eastern chipmunks.

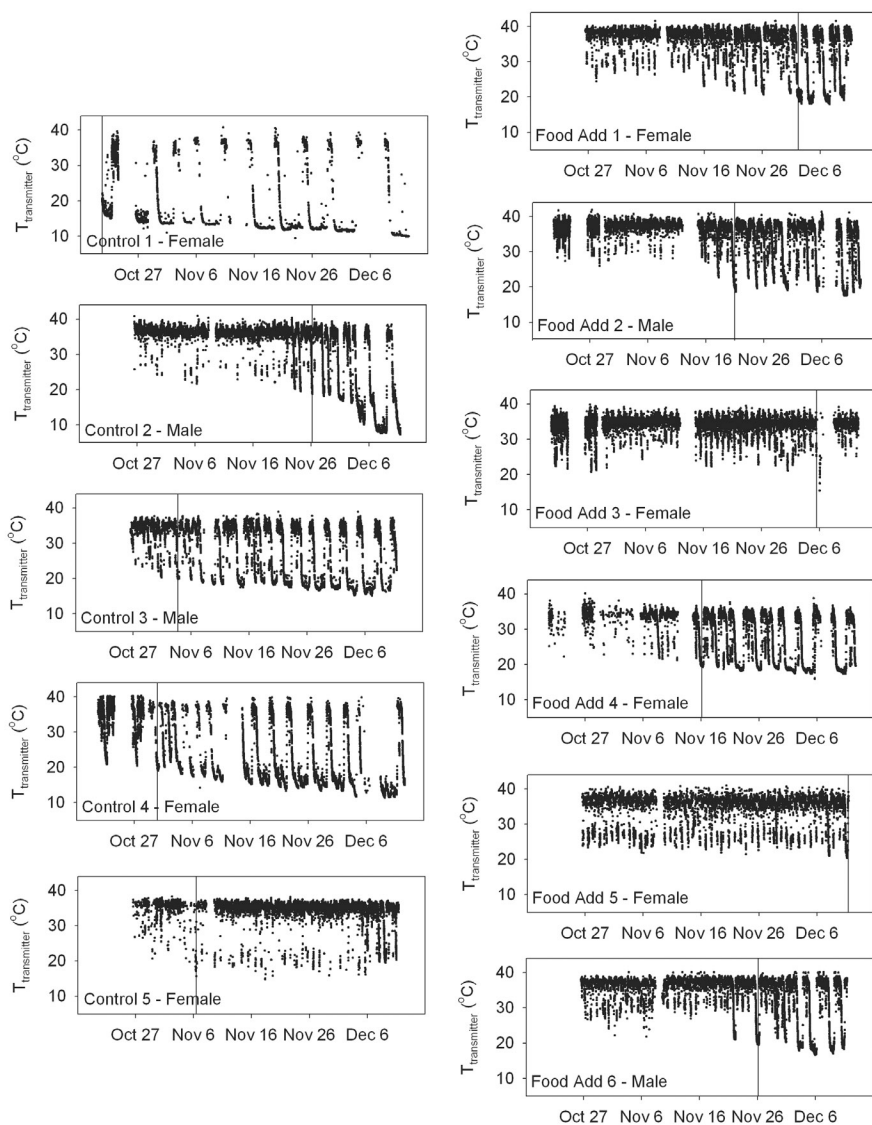


Fig. 3. Post-immurgent torpor patterns of five control and six food supplemented chipmunks as recorded from neck-mounted, temperature-sensitive transmitters. Vertical lines indicate first occurrence of torpor bouts below where baseline $T_{\text{transmitter}}$ was $< 20^{\circ}\text{C}$.

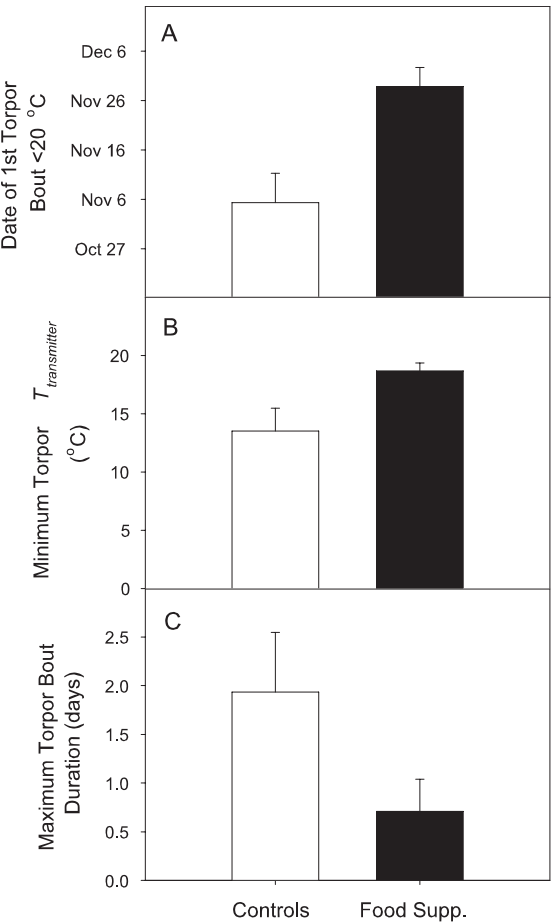


Fig. 4. Effect of energy reserves on post-immigrant torpor expression by eastern chipmunks. Means are presented ± 1 SE, and in each case $n=5$ for controls and $n=6$ for food supplements. (A) See Fig. 1 and Fig. 3 captions for description of treatments and response variable. (B) Minimum Torpor $T_{transmitter}$ is the minimum baseline temperature reached during the deepest torpor bout. (C) Maximum Torpor Bout Duration is the longest recorded interval from the start of re-entry into torpor to the start of the arousal out of torpor.

In contrast to the lack of a state-dependent activity response, torpor expression following immersion was clearly influenced by the food supplementation. Individuals with experimentally enlarged food hoards initiated deep torpor bouts much later and were characterized by shallower and shorter torpor bouts than controls. These results are consistent with previous evidence for torpor avoidance in eastern chipmunks based on periodic, instantaneous sampling of transmitter temperatures, and thus provide an important confirmation of this result with continuous sampling of torpor expression. The effect sizes reported here are not as strong as those reported by Humphries et al (2003b), but this may result from between-study differences in natural food availability and the diversity of food types provided. Munro et al. (in prep.) have recently

demonstrated that both hoard size and hoard composition influence torpor expression by free-ranging eastern chipmunks. In any case, the torpor patterns of control and food supplemented chipmunks in this and other studies emphasize the extreme plasticity of torpor expression in this species. As illustrated in Fig. 3, one control chipmunk expressed early-winter torpor bouts exceeding four days in length with a baseline temperature of 8° C, while most food supplemented and one control chipmunk had body temperature patterns more characteristic of daily torpor than prolonged torpor (Fig. 3). The fact that chipmunks with large energy reserves do not avoid torpor per se, but rather appear to avoid deep, prolonged torpor, suggests that the costs of torpor may be more severe at low body temperatures. Furthermore, these results cast additional doubt on whether prolonged torpor and daily torpor are discrete physiological phenomenon, or simply empirical clusters along a continuum of state-dependent physiology (Wilz and Heldmaier, 2000).

Acknowledgements

We thank Corentin Bohl and especially John Humphries for excellent assistance with fieldwork. This research was supported by NSERC Discovery and Research Tools & Instruments grants to MMH.

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Seasonal Timing of Reproduction and Hibernation in the Edible Dormouse (*Glis glis*)

CLAUDIA BIEBER AND THOMAS RUF

Research Institute of Wildlife Ecology, University of Veterinary Medicine, Vienna, Austria

Abstract. Edible Dormice (*Glis glis*¹) hibernate for extremely long periods (up to > 8 months), although they inhabit temperate zone areas with moderate climatic conditions. Juveniles are born late in the active season (August) and have little time for growth and prehibernation fattening. Compared to other hibernators with single litters per year, this seasonal onset of reproduction is extremely late. However, we found no evidence for exceptionally high growth rates in juvenile dormice. Our field observations indicate that juveniles instead respond to the limited time for fattening in fall by a significantly shorter hibernation period than adults. Evidence from this and previous studies indicates that this peculiar temporal pattern of hibernation and reproduction is due to a specialization of dormice on tree-seeds, namely beechnuts, which reach highest mass and energy content only late in the vegetation season. We found that dormice after emergence in spring anticipate future food availability and may, in years without beechnuts, entirely skip gonadal growth and reproduction. Skipping of reproduction results in increased probabilities to survive until the next year and thus maximizes lifetime reproductive success.

1. Note that *Glis glis* is the correct scientific name of the edible dormouse, although it was temporarily named *Myoxis glis*.

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

Introduction

The timing of reproduction is a sensitive process in many species. However, mammalian hibernators in seasonal fluctuating environments often face extreme limits caused by the short duration of their active season. Within a few months they have to undergo gonadal development, because hypothermia and sexual competence are not compatible (e.g., Barnes et al., 1986); establish territories and mate; raise young; and restore fat or food stores for the next hibernation season. Theoretically, juvenile survival chances should be optimized by reproducing early in the active season, which ensures sufficient time for juveniles to grow and to accumulate energy reserves prior to their first hibernation season. Indeed, many hibernators raise only one litter per year and it is well documented that early litters have a higher probability of survival (Armitage et al., 1976; Murie and Boag, 1984; Sauer and Slade, 1987). It is the more astonishing then that edible dormice (*Glis glis*) in their northern distribution range (Germany, Austria, northern Italy) give birth to a single litter very late in the year—during August—about three months after the start of the vegetation period (Vietinghoff-Riesch, 1960; Pilastro, 1992; Bieber, 1995; Schlund, 1996; Pilastro et al., 2003; Fig. 1). To our knowledge there is no other hibernating species with a comparably delayed seasonal onset of reproduction. Hibernation in dormice is well documented (Wyss, 1932; Vietinghoff-Riesch, 1960; Wilz and Heldmaier, 2000) and lasts about eight months. Dormice are not known to store food before hibernation (Schacht 1886; Vietinghoff-Riesch, 1960; Morris and Hodless, 1992) and thus appear to rely solely on their body fat reserves to survive hibernation. The temporal patterns of reproduction and hibernation are caused by the strong relation between reproduction and the availability of beechnuts and acorns in this species. In fact, in years with a lack of mast of beech (*Fagus sylvatica*, which is the most common tree species in deciduous forests in central Europe) dormice fail to reproduce and very few or even no juveniles are detectable (Bieber, 1995, 1998; Schlund, 1996; Schlund et al., 2002; Pilastro et al., 2003; Fietz et al., 2004). In the present study we investigate the causes of this seasonal timing of hibernation and reproduction in dormice more closely and discuss its consequences for the distribution and specific life history traits in this species.

Material and Methods

Field Study I

One part of this study was carried out near Marburg, central Germany. We captured dormice in wooden life-traps set on branches at a height of 1–2 m in

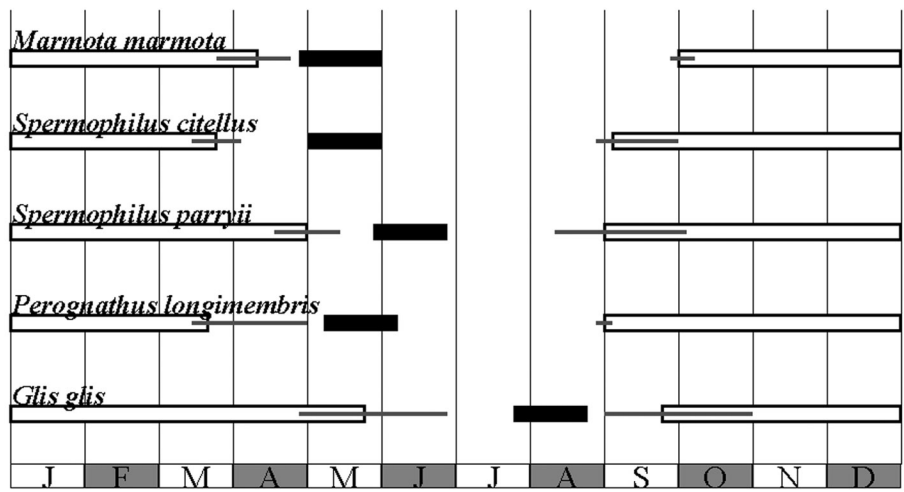


Fig. 1. Seasonal temporal patterns in some hibernators. Black bars: time of birth of litters, white bars: Average time of hibernation, grey lines: variability in emergence and immergence. *M. marmota*: Arnold, 1999, F. Frey-Roos pers. com.; *S. citellus*: Millesi et al., 1999, S. Huber, pers. com.; *S. parryii*: Buck and Barnes, 1999; *G. glis*: Bieber, 1995, this study. Note that *P. longimembris* emerge very late and skip reproduction in certain years (Kenagy and Bartholomew, 1985).

the years 1992–1993 and 1995–1996. The study site within a deciduous forest (altitude 300–350 m) had a total size of 2.5 hectares. Animals were trapped for three consecutive trapping nights (12 h each) twice a month during the active season (April to November). We never captured dormice in the first or the last seasonal trapping interval, which indicates that our trapping schedule allowed us to determine the onset and offset of the active season of dormice. All dormice captured were registered (age, sex, weight, trap location), and newly captured animals were marked by ear tattooing (5 mm tattoo-pliers, Hauptner, 80336 Munich, Germany) before being released at the capture point. For more details on the study site, age estimation, and trapping methods see Bieber (1995, 1998).

Field Study II

To investigate the relation between occurrence of beechnuts (mast) and reproduction in dormice, we analyzed data collected during a long-term study carried out in the Vienna Forest, Austria (F. Schieferdecker, pers. comm.). In this

study the number of dormice in tree-mounted nest boxes (approximately 200 nest boxes per year in a 650 hectares deciduous forest area; altitude 400–550 m) were counted each year between October and November, from 1984 until 2002. Since dormice occur in high numbers during late autumn only in years with successful reproduction (Vietinghoff-Riesch, 1960; Hönel, 1991; Schlund, 1996; Fietz et al., 2004), we assumed that the majority of dormice counted at this time of the year were juveniles (although the age of animals was not registered). Direct measurements of beech mast were not available for the study area. Instead, we used the annual sum of beech pollen density (# of pollen per m³, measured at a distance of 40 km from the study site; S. Jäger, pers. comm.) as an indication for the yearly production of beechnuts. Since beech is known to have synchronized mast seeding over a wide geographical range (Jenni, 1987) we assumed that beech pollen data measured 40 km apart reliably reflected the pollen density in our study site. The complete synchrony of beech mast cycles between Marburg (Bieber, 1998) and Tübingen (in southwest Germany, Schlund et al., 2002), approximately 400 km apart, further justified this assumption. In addition, we determined the chemical composition of beechnuts (i.e., fat, protein, and carbohydrate content) in July and August 2003 using methods as specified in Hackländer et al. (2002).

Laboratory Studies

Dormice were kept in groups ($n = 5\text{--}20$ each) in three enclosures (each 6 x 4.5 x 3 m) under natural climatic conditions at the Research Institute of Wildlife Ecology, Vienna (Austria). Large branches and wooden nest boxes were placed in the enclosures to deliver shelter and opportunity to climb. Dormice were bred in the years 1999 and 2003. We did not disturb litters ($n = 3$) in 1999 until three weeks of age. Afterwards we measured body mass of juveniles once a week until end of October. In 2003, body weights of juveniles were determined twice a week over a period of five weeks, starting on the day of parturition. Food pellets (Altromin rodent chow [1314 FORTI, fat content: 5.0%, protein content: 22.5%], Altromin, Germany) and water were given *ad libitum*. After weaning, juveniles were additionally fed with sunflower seeds, acorns, and apples.

Statistical Analyses

Statistical analyses were carried out using S-Plus 6.1 (Insightful Cooperation, Seattle, USA). Means are given \pm one standard deviation. To test for differences in the number of males, females, and yearlings trapped at certain times of the

year we used χ^2 tests. Spearman's rank correlation coefficient was computed to test if pollen-production of beech (*Fagus sylvatica*) and oak (*Quercus spec.*) and the number of dormice in nest boxes in autumn were correlated over the years. Pearson's product moment correlation was used to test the relation between the percentage of sexually active adults (males: large, tangible testis during mating time; females: visible nipples) per year and recapture frequency (as an indication for total activity) within that year. We used Cormack-Jolly-Seber models (program MARK 3.2; White and Burnham, 1999) to estimate local survival and recapture probabilities. Goodness-of-fit tests of the full model and model selection procedures were carried out as described in detail in Pilastro et al. (2003).

To calculate expected litter mass at birth and expected individual mass at weaning in mammals, we used Peters' (1983) modification of an allometric relation determined by Blueweiss et al. (1978), and Millar's (1977) equation, respectively. For these calculations we used mean weights of adult females after emergence from hibernation. For comparisons, litter mass and weaning weight data from some other hibernators with a single litter per season were obtained from the literature (Arnold, 1999; Buck and Barnes, 1999; Huber et al., 2001; Kenagy and Bartholomew, 1985; E. Millesi, F. Frey-Roos, pers. comm.).

Results and Discussion

Seasonal Timing

Dormice are nocturnal inhabitants of deciduous forests with an adult body mass of 100–130 g after emergence from hibernation (Bieber, 1998). In spite of their strong arboreal life during the active season, dormice hibernate underground at a depth of 30–80 cm (Vietinghoff-Riesch, 1960). At our study site in Germany yearlings started to emerge at the end of April, adult males emerged during May, and adult females were first trapped approximately one month later, during June and July. This difference in the timing of emergence between adult males and females was statistically significant ($\chi^2 = 12.8$; $df = 1$; $p < 0.001$). Mating occurred between end of June and beginning of July. In late July through mid August, after 28–30 days of gestation, females give birth to 1–11 juveniles (average 5; Vietinghoff-Riesch, 1960; Pilastro 1992, Pilastro et al., 2003; Koenig, 1960). In Germany, dormice started to hibernate at the end of August (adults), but juveniles were still captured above ground until the end of October. The average time lag between last captures of individuals in fall and their first capture in spring was 300 ± 20 d in adult males ($n = 39$), 316 ± 12 d in adult females ($n = 24$), and significantly shorter (290 ± 20 d) in juveniles ($n = 38$, $F_{100,1} = 9.24$,

$p < 0.01$). This duration (of 10 months in adults) probably exceeds the actual hibernation period since we trapped at two-week intervals only, and because expectedly, animals were less prone to capture after prehibernation fattening in fall.

It is known that dormice typically hibernate for around eight months in Germany (Vietinghoff-Riesch, 1960; Bieber, 1998; Fietz et al., 2004), but slightly less (seven months) in the warmer climate of Italy (Pilastro et al., 2003). It should be noted that this extremely long duration of hibernation is, to our knowledge, matched only by a single other species, *Spermophilus parryii*, which lives under much more extreme, arctic conditions and faces a much shorter vegetation period (Fig. 1). However, in dormice the “effective” vegetation period may be similarly brief, because in this species, reproduction is tightly linked to the availability of certain seeds, in particular beechnuts (Bieber, 1998; Schlund et al., 2002; Pilastro et al., 2003; Fietz et al., 2004). Pilastro et al. (2003) observed a strong dependence of dormouse reproduction on beech mast in northern Italy, and argued that the dormouse-beech system represents an example of prey-predator spatiotemporal synchronization in forest areas in which beech is the dominant tree species. Our measurements of the composition of beech seeds at different stages showed the highest fat (and thus, energy) content in early August (43.1% on August 9), just in time with parturition in dormice, and still available up in the trees. A few weeks earlier (July 19) beechnut fat content was much lower (15.8%). Hence, the late emergence and delayed timing of parturition clearly represent adaptations of dormice to the seasonal time course of beechnut (and possibly acorn or other tree seed) development. As a consequence, juveniles born in August have only about two months to store fat reserves for their first hibernation.

Beechnuts provide an excellent food source for both juveniles after weaning (notably, their high protein content of 27.02% [August 9] should facilitate growth) and for lactating females. Accordingly, we found no evidence for significant decreases in body weight among adult females (mean weight: 106 ± 11 g; $n = 23$) during lactation in August on our study site. However, as pointed out by Pilastro et al. (1994), females appear to follow different reproductive strategies, depending on their body condition: females in good condition breed early—within a time window of approximately 20 days—lose weight during lactation, and wean young when food abundance is optimal. Litters of these females, born early, reach maximal prehibernation weights. Females in poor condition delay parturition and lactate during the time of peak food supply. This strategy leads to high weaning weights of litters but lower prehibernation weights due to the

limited time span available for growth and the depletion of food resources. In those “late” females, no body mass loss was observed, indicating that the high quality of food may compensate directly for high rates of energy expenditure during lactation (Pilastro et al., 1994).

Juvenile Growth Rates

Since dormice are born very late in the season compared to other hibernators with single litters, one could expect extremely high growth rates in juveniles because (1) time for pre-hibernation fattening is short and (2) beechnuts provide an excellent food quality. Therefore, we measured growth rates of juveniles under laboratory conditions.

We were able to detect litters at the day of parturition and hence exactly determine the age of young in our enclosure in four cases. Total litter birth weights were 4.5, 21, 23.5, and 26 g in litters with 1, 5, 6, and 9 juveniles respectively. The average birth weight per juvenile was 3.7 ± 0.6 g ($n = 21$). Mean weight gain per individual was 1.28 g d^{-1} within the first 30 days of life (Fig. 2). In juveniles captured in the field at the beginning of September (i.e., at an age of

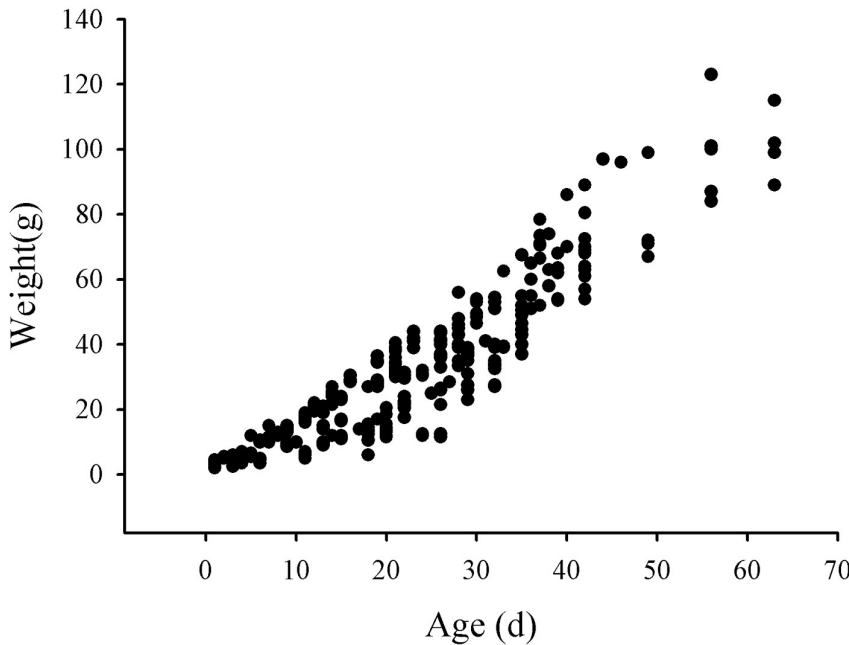


Fig. 2. Growth in juvenile dormice ($n = 13$).

approximately 30–40 d) mean weight was $39.3 \text{ g} \pm 5.9 \text{ g}$ ($n = 48$). At the end of the active season (October) juveniles reached a body weight of $86.9 \text{ g} \pm 18.5 \text{ g}$ ($n = 22$) at an age of approximately 50–70 days. These field data are well in the range of the measurements shown in Fig. 2, which indicates that the weights and growth rates observed in the laboratory were comparable to those of wild dormice.

Comparing juvenile weights with data from several other hibernators (species with one litter per year, see Fig. 1), we found that in all species birth weights were at or slightly below the values expected from the allometric curve for mammals (Blueweiss et al., 1978; Peters, 1983). Weaning weights, on the other hand, were higher than expected for mammals of that size (Millar, 1977) in three species, namely *P. longimembris* (20% above expectation), *G. glis* (+24%) and *S. parryii* (+119%) suggesting that growth rates in hibernators may be generally high. The average individual weight gain in dormice until weaning was large (10.4 x birth weight within 30 days) but not out of range compared to other hibernators (factors 7, 10.5, and 7 in *M. marmota*, *S. citellus*, and *P. longimembris*, respectively, all within 28–30 days). Dormouse growth rates did not match that of arctic ground squirrels, which appear to gain 22 times their birth weight in 28 days. One explanation for this difference is that energy expenditure during hibernation in dormice is arguably less than in the Arctic ground squirrel, for instance, which has to hibernate under extreme environmental conditions (at burrow temperatures below -20°C) that require high rates of thermoregulatory heat production even in deep hibernation (Buck and Barnes 1999). Further, dormice are arboreal and should avoid carrying too much excessive body fat reserves that may impair agility and increase predation risk. Hence, for juvenile dormice the best strategy may well be shortening of the hibernation season since food - even if at lower quality than seeds—is usually available early in the next year. This may explain our above observation that juvenile dormice are the first to emerge and that hibernation is significantly shorter than in adults (see also Vietinghoff-Riesch, 1960; Bieber, 1995; Schlund, 1996).

Skiping of Reproduction: Year-to-year Fluctuations

During our field study in Germany we observed a lack of mast on our study site in the years 1993 and 1996. In contrast, 1992 was a full mast year (80%–100% of beech trees produced seeds) and in 1995 about 25% of the beech trees produced seeds. These differences in beechnut availability were paralleled by the presence or absence of reproduction on the entire study site in those years:

Juveniles occurred in 1992 ($n = 136$) and 1995 ($n = 94$) only. We did not capture a single juvenile in our life-traps in the years 1993 and 1996. Since dormice readily breed in artificial nest boxes, we used nest box controls ($n = 20$) on our study site and the surrounding forest to validate our trapping results. Using this method we also found litters in the nest boxes only in the years 1992 and 1995 (in early August), and complete reproduction failures in 1993 and 1996.

Interestingly, in males there was some evidence for effects of reproductive state on recapture frequency, i.e., probably their general level of activity. We related recapture frequency of male dormice per year with percent sexually competent males (during July) and found a tendency for fewer recaptures per adult male in years with fewer sexually competent males ($r = 0.947$, $df = 2$, $p = 0.0526$). While we observed no sexually competent males in 1993, we captured 100%, 96%, and 60% males with tangible testes in 1992, 1995, and 1996 respectively. Recapture frequency was lowest in 1993 (1.90 ± 0.99) and higher in the years 1992 (3.17 ± 1.71), 1995 (2.77 ± 1.98), and 1996 (2.74 ± 1.84). Among adult females there was no correlation between percent of lactating females and recapture frequency ($r = 0.341$, $df = 2$, $p = 0.6585$).

It should be noted that the phenomenon of skipping of reproduction is not restricted to particular populations. As a matter of fact it now seems that dormice in their northern range of distribution generally reproduce only in years with mast of large seed trees, mainly beech and possibly also oak (Bieber 1995, 1998; Schlund, 1996; Schlund et al., 2002; Pilastro et al., 2003; Fietz et al., 2004). This pattern of skipping of reproduction was also apparent in our analysis of data from an 18-year-long nest box control study in the Vienna Forest, Austria. There was a significant correlation between the number of dormice found during autumn (Sept./Oct.)—which is indicative for reproductive output (Vietinghoff-Riesch, 1960; Bieber, 1995; Schlund, 1996; Fietz et al., 2004)—and the annual sum of beech pollen per cubic meter ($Z = 3.0242$, $\rho = 0.7345$, $p = 0.0025$, Fig. 3). Pollen density of other tree species like oak (*Quercus spec.*) was not correlated with the number of dormice in the nest boxes ($Z = 1.3119$, $\rho = 0.319$, $p = 0.1896$). Interestingly, we found a correlation between beech pollen concentration (flowering) and reproduction in dormice, although one should expect that after flowering a number of ecological factors might influence fall beech seed production (e.g., weather, insect damage).

Taken together, these results indicate that edible dormice belong to those rare species that are highly adapted to strong year-to-year fluctuations in food availability. These fluctuations are largely unpredictable. Years of beech mast occur

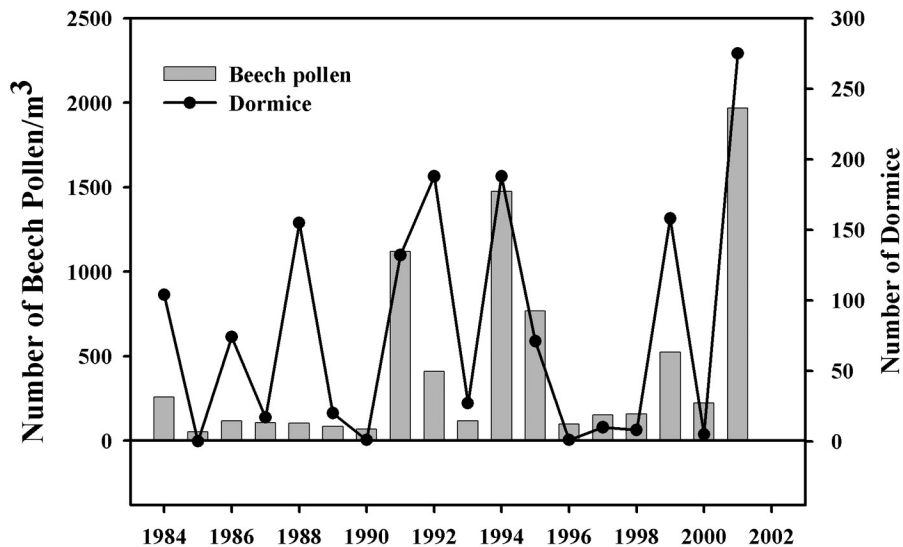


Fig. 3. Time course of abundance of juvenile dormice and beech pollen (# of pollen per m³) in the Vienna Forest (Austria).

randomly, without a stable periodicity (Ruf et al., unpublished). The only regularity in the temporal pattern of beech masts is that years of full mast are always followed by a complete lack of seeds in the subsequent year. Due to the overall unpredictability of seed production dormice apparently decide each spring whether or not they will invest in reproduction in any particular year (e.g., the availability of young beech seeds may serve to estimate future food supply). Interestingly, this also applies to males, which either grow testes or remain sexually quiescent even before the emergence of females (Bieber, 1995). This decision by males was not based on poor body condition, as occasionally males were found to be in a somewhat better condition in spring in years when reproduction was skipped (Bieber, 1998; Pilastro et al., 2003). It seems therefore that male dormice do not emerge from hibernation with a body mass below a threshold preventing gonadal development. In this respect dormice differ from other hibernators (e.g., Barnes, 1984). Recently, Fietz et al. (2004) found that sexually incompetent males, after emergence from hibernation in spring, used daily torpor more frequently than sexually active males. However, it is unclear whether males in certain years are forced to minimize energy expenditure via torpor, which prevents spermatogenesis and gonadal recrudescence or if, vice versa, dor-

mice use environmental signals (e.g., the presence of seed buds) to predict future food availability. In the latter case, the occurrence of daily torpor would not be the cause but only an epiphenomenon of gonadal quiescence. In any case, the regulatory mechanisms responsible for reproduction skipping in unfavorable years are not perfect: In 1996 we observed that 60% of all males had fully developed gonads, but no juveniles were born in that year. This indicates that, at least among males, there is large phenotypic variability in the response to identical environmental conditions. Females seemed to independently decide whether to invest in reproduction or not in any year, unaffected by male responses.

One could expect that dormice compensate for years with reproductive failures by living long. A capture-recapture study in Italy has shown recently that free-living dormice indeed can reach an average longevity of nine years, which is clearly higher than expected for a mammal of this size (Pilastro et al., 2003). In this Italian population, local survival was generally high in both reproductive and nonreproductive years. In our study population in Germany (under rougher climatic conditions) we found a direct relation between reproductive effort and yearly survival probability. In both sexes local survival probability was 87% (0.87 ± 0.37) in the interval 1993 to 1994, after a complete skipping of reproduction. This was higher than the average survival probability in adults following reproductive years (0.64 ± 0.14). We believe that part of the increased survival rates in nonreproductive years is related to lower general activity, which leads to lower recapture rates, as observed in our population. Expectedly, reduced territorial and general locomotor activity should decrease both energy expenditure and predation risk. Whatever the underlying pathways, the extreme year-to-year variation in food resources and the high specialization of dormice on these resources provide an interesting natural experiment that demonstrates a fundamental life-history trade-off between reproduction and survival (Williams, 1966).

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Reproduction and Hibernation in Females: A Comparison of Two Sympatric Ground-Dwelling Rodents

EVA MILLESI, ILSE E. HOFFMANN, ANNA ASCHAUER, AND CLAUDIA FRANCESCHINI
University of Vienna, Institute of Zoology, Vienna, Austria

Abstract. In this study we compared the timing of hibernation and reproductive output in two sympatric mammal species, the European ground squirrel (*Spermophilus citellus*) and the European hamster (*Cricetus cricetus*). Female ground squirrels only have one litter per season whereas hamsters can rear up to three litters. *S. citellus* have a shorter active period than the hamsters. Despite of these differences there were similar effects of timing of the active season on reproductive output in both species. Early onset of activity and reproduction resulted in higher numbers of offspring. Body mass at emergence from hibernation was not significantly related to reproductive output in neither species. Females with high reproductive output prolonged the active season and entered hibernation later than less successful individuals. Body mass at the onset of hibernation was not related to previous offspring number, indicating that successful females can compensate the costs of reproduction by remaining active until late in the season. The similarities in these two species demonstrate the importance of timing of the active season and hibernation on reproduction. This in turn is surprising in light of obvious differences in hibernation patterns and reproductive strategies.

Introduction

Analyses of individual life-history strategies document trade-offs that animals make as they allocate critical resources to reproduction and/or survival. In hibernating mammals, the timing of reproduction and its energy allocation are rigidly controlled. The result is a very strict temporal pattern of various seasonal

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processes like mating, reproduction, molt, and prehibernatory fattening (Heller and Poulson, 1970; Michener, 1984; Millesi et al., 1998, 1999a). Time spans for these activities are short, and energy demands increase with the need for expedience and efficiency. As a result, most obligate hibernators like the European ground squirrel (*Spermophilus citellus*) are only able to raise one litter per year (Millesi et al., 1999b; Phillips, 1984). Carry-over effects of reproductive costs from one year to the next have been documented in *S. citellus*. Long-term studies have demonstrated that high maternal effort in one year negatively affected reproductive output in the next (Huber et al., 1999, 2001).

Another kind of solution to this time and energy dilemma has been found in other hibernators like the European hamster (*Cricetus cricetus*). These animals do not depend on their body fat reserves for hibernation. Instead, they build up food caches, which can be used during winter (Niethammer, 1982). The advantages of this strategy are the potential timesaving in foraging and the unlimited nature of extra-corporal energy allocation. As a result, time and energy constraints on reproduction are less severe. Animals using this strategy are able to maintain a higher reproductive output than other hibernating species (Grulich, 1986; Niethammer, 1982). *C. cricetus*, for instance, has up to three litters per season and early puberty in both males and females (Grulich, 1986). An additional timesaving adjustment can be made by overlapping ovulation and lactation patterns. Female European hamsters can go into a postpartum estrus and become pregnant while still lactating (Franceschini, 2002; Grulich, 1986). Nonetheless, there is a high degree of inter-individual variation in puberty, gestation length, number of litters, and litter size, which probably reflects the interacting effects of condition, development, and environment (Franceschini and Millesi, 2001; Grulich, 1986; Niethammer, 1982; Weidling and Stubbe, 1997).

European ground squirrels and European hamsters show a wide overlap in their distribution in the eastern part of Austria (Spitzenberger, 2001; Hoffmann et al., unpublished data). In Vienna, both species have been observed to be sympatric in a number of areas south of the river Danube. They have similar habitat requirements concerning soil quality, arid vegetation, and low groundwater tables, although hamsters seem to tolerate a wider range of suitable habitats than ground squirrels, like agricultural areas.

In this study we compared the effects of the timing of the active season and emergence body mass on reproductive performance of individual females of both species living in similar habitats. In addition, we examined potential con-

sequences of reproductive output on female condition and the timing of the subsequent hibernation period.

Methods

European ground squirrels (*Spermophilus citellus*) were investigated in a suburban area (4 ha) in Langenzersdorf, close to Vienna, Austria. In this study, data of the years 1997–1999 were used. The studied European hamster population (*Cricetus cricetus*) lived in the southern part of Vienna in a similar habitat (2 ha). Data of two seasons, 2001 and 2003, are reported.

Animals of both species were captured with live traps (Tomahawk Trap Co.) baited with peanut butter. All individuals were permanently marked with subcutaneously injected PIT- tags (Indexel or Data Mars), and fur-marked with a commercial hair dye for distant recognition. Detailed descriptions of the capture techniques appear in Millesi et al. (1999a) and Franceschini and Millesi (2001). Animals were weighed (± 0.5 g, Sartorius laboratory scale) at each capture. Emergence mass was measured in individuals that were captured within three days after their first emergence from the hibernacula. Immergence body mass was recorded if an individual was captured within four days before its last observation above ground in late summer (Millesi et al., 1999a). Reproductive status was defined on the basis of vulval and teat development. Vulval development was classified as small/closed to swollen/open on a three-point scale in both species. Teat development, milk secretion, and body mass were documented as indicators for gestation and lactation. In ground squirrels, teats were swollen and darkly pigmented during pregnancy. When lactation started, teats lightened, were larger, and milk excretion could be induced (Millesi et al., 1999b). In the hamsters, teats start to swell during pregnancy and are red and less swollen during lactation (Franceschini, 2002). The use of these data as time points of seasonal activities was supported by sudden body mass changes after parturition or a slow increase after weaning. Litter size was defined as the number of juveniles that emerged from a female's breeding burrow. Only litters whose mothers were known were used for analyses. Litter emergence was defined as the first emergence of juveniles from the female's burrow.

Scan-sampling techniques were carried out at irregular intervals each day, and the daily scans were supplemented with all additional occasional sightings not recorded during scans (Millesi et al., 1999a; Pfeifer, 1982). Checklists of active individuals in the population were maintained daily after snowmelt until the last individual had emerged from hibernation and from the beginning of July until

the last individual had disappeared. Checklists were used to produce a saturation curve showing that by scanning 1 ha five times per day, > 90% of marked individuals could be identified.

Results

Annual Cycle

Initially, we compared the timing and duration of hibernation and the active season in individuals of both species to determine the time frame for reproductive and other seasonal activities. Female European ground squirrels started surface activity between late March and mid April (mean: 04/04, SD: 7.2d, $n = 39$). The active phase lasted for about 4.5 months, and females immersed into hibernation during August (mean 08/15, SD: 20.7d, $n = 38$). Accordingly, female ground squirrels hibernated for seven to eight months (mean: $229.8d \pm 10.4$, $n = 13$). The active period of female European hamsters was longer than that of ground squirrels. They emerged in late March (mean: 03/29, SD: 6.1d, $n = 11$) and were active until late September (mean: 09/25, SD: 14.5d, $n = 7$). To date, we have no accurate data on hibernation duration of individual females.

Reproductive Timing and Output

To investigate the importance of timing of the active season on reproductive performance of individual females, we compared emergence dates and the reproductive output of ground squirrels and hamsters. In both species we found significant relationships between emergence from hibernation and the onset of reproduction. In ground squirrels, timing of female emergence from hibernation was positively correlated with timing of natal emergence of her litter (Fig. 1a). In addition, litter size was related to litter emergence date in that earlier litters were larger than later ones ($r_s = -0.49$, $p < 0.05$, $n = 30$). Accordingly, females that had hibernated for a shorter period had larger litters in the subsequent season (Fig. 1b).

In European hamsters we found similar relationships between reproductive timing and output. Female emergence from hibernation and the onset of gestation were significantly correlated (Fig. 2a). The number of litters as well as the overall offspring produced in one season were negatively correlated with the emergence date of the mother (Fig. 2b, number of litters: $r_s = -0.87$, $p < 0.05$, $n = 7$).

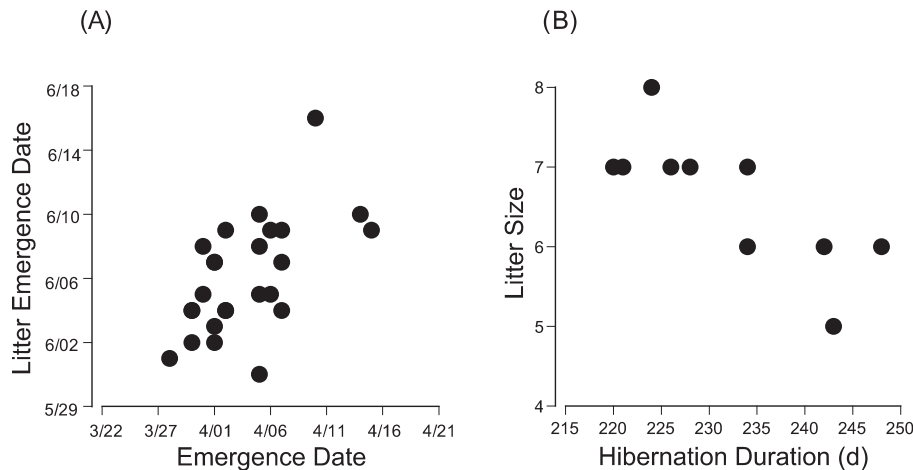


Fig. 1. Timing of the active season and reproductive output in European ground squirrels. (A) Female emergence date from hibernation was positively correlated with the first emergence of the litter from the natal burrow ($r_s = 0.57$, $p < 0.001$, $n = 35$); (B) Litter size was negatively correlated with the duration of the previous hibernation period ($r_s = 0.60$, $p < 0.05$, $n = 15$).

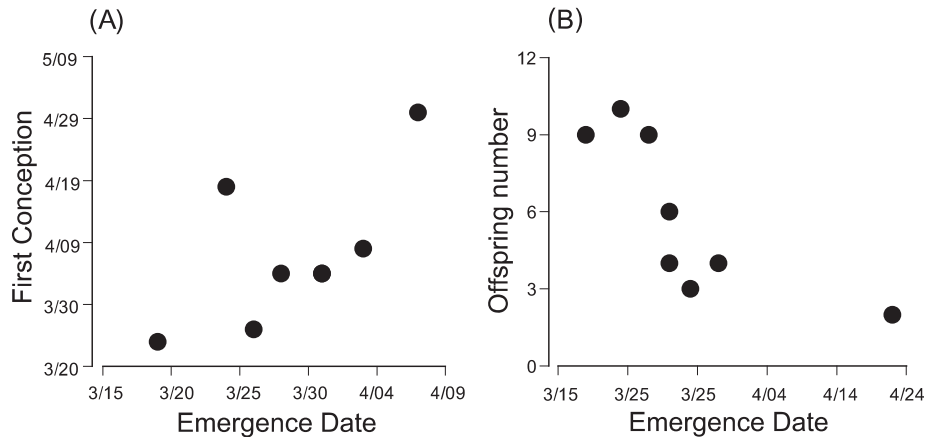


Fig. 2. Emergence from hibernation and reproduction in European hamsters. (A) The onset of the first pregnancy and female emergence from hibernation ($r_p = 0.79$, $p < 0.05$, $n = 8$). (B) Female emergence date and offspring number ($r_p = -0.83$, $p < 0.05$, $n = 7$).

Consequences of Early Reproduction

Individual differences in reproductive output could cause differential maternal effort and therefore may affect the condition of the mother and/or the timing of the subsequent hibernation period. We compared reproductive output in a season to the subsequent onset of hibernation. Significant patterns were found again in both species. Female ground squirrels that emerged early had a longer active period compared to the later ones (emergence date : immergence date, $r_s = 0.33$, $p < 0.05$, $n = 38$). There was no significant relationship between litter size and subsequent immergence into hibernation. On the other hand, female hamsters with high reproductive output were found to begin the subsequent hibernation later in the season than the less successful ones ($r_p = 0.96$, $p < 0.03$, $n = 6$).

To investigate direct conditional effects on the timing of hibernation and reproduction, we compared emergence mass with reproductive onset and output. We found no significant correlations among these parameters in either species ($p > 0.1$ in all cases). In addition, reproductive output did not seem to affect the females' condition shortly before hibernation. In female ground squirrels, emergence and immergence body mass were positively correlated ($r_p = 0.78$, $p < 0.04$, $n = 11$). However, no significant relationships between offspring number and immergence mass were found in either of the two species ($p > 0.3$ in all cases).

Discussion

The presented data underline the importance of timing effects in hibernating animals. Although ground squirrels and hamsters differ in many aspects of hibernation and reproductive strategies (Wassmer and Wollnik, 1997; Wollnik and Schmidt, 1995), the early onset of the active season and correspondingly the chance to start reproductive activities as soon as possible seems to be a determining factor for reproductive success in both species.

In ground squirrels and hamsters, litter sizes decreased with season. European ground squirrels give birth to only one litter per season, and early reproduction may reduce the time constraints for lactation and prehibernatory fattening (Phillips, 1984). Huber et al. (2001) have shown that in *S. citellus* larger litters were nursed for longer than smaller ones. Juveniles of small litters were heavier than those of larger litters at natal emergence, but the latter were able to catch up due to a prolonged lactation period.

In hamsters, early emergence seems to allow a female to have more than one litter per season. In our study population only two of the focal females gave birth three times a year and some individuals (56%) had only one litter.

Although the litter sizes of individual females decreased with season (Tauscher et al., unpublished data), the number of litters determined the overall reproductive output in the studied seasons. This opportunistic reproductive strategy has been documented in a number of studies and can lead to extreme population increases when environmental conditions are favorable (Grulich, 1986). Potential effects of sequential litters on maternal investment and reproductive success in the following year will be the focus of future studies.

Female condition in terms of body mass at emergence did not seem to be a determining factor for reproductive success in the studied populations, although it has been shown that female ground squirrels in very poor condition at emergence usually delay reproduction and have none or very few offspring (pers. observation). There is also evidence that very light individuals emerge earlier because of a lack of body reserves, but in the years reported in this study this was not the case. One reason might be that only individuals in a sufficient condition survived until hibernation (Hoffmann et al., 2003). Over-winter mass loss has been shown to be very constant in these ground squirrels, so that an individual's condition before winter also determines its emergence mass (Millesi et al., 1999a).

Although the two species have similar strategies to deal with time constraints, they seem to differ in how they compensate the costs of reproduction. Ground squirrels have only one litter per year and should invest as much as they can afford energetically. Early-reproducing females have larger litters, but they also can spend more time for lactation than the later ones. The timing of the subsequent seasonal processes like molt and prehibernatory fattening has to be quite strict because food quality starts to decrease in late July (Quinn, 1988; Pieta and Dittami, 1997).

Female hamsters that produce more than one litter have a longer reproductive period and therefore postpone the onset of hibernation. This seems to be quite variable between years, since in 2003 all adult females had disappeared by late September whereas in 2001 we were able to observe and capture active individuals in early November. Hamsters therefore appear to be more flexible in the timing of hibernation than the ground squirrels.

The possibility to reproduce early in the season is probably related to follicular development where time is an essential component. Follicular growth is inhibited during gestation and lactation. Hence, the preparation for reproduction in the next season can occur after weaning, during hibernation, or in the following spring. In ground squirrels, histological data on ovaries collected shortly before and after hibernation indicate that follicular development is initiated in

summer after weaning and continued until hibernation (Millesi et al., 2000). This could save important time for reproduction in the next season. In hamsters we are presently investigating these interactions. Absolute body mass and reproductive effort may have only minor or indirect effects. The possibility exists, however, that other nutritional components like essential minerals, amino acids, or polyunsaturated fatty acids may play a role here.

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How the Photoperiod Times the Annual Reproductive and Hibernation Cycles

P. PÉVET, M. SABOUREAU, AND P. KLOSEN

Neurobiologie des Rythmes, Université L. Pasteur, Strasbourg, France

Abstract. In mammals, several physiological functions (such as breeding, moult, and hibernation) exhibit seasonal changes important for survival during the cold period. In temperate regions, photoperiod is the most reliable environmental cue used to drive these seasonal adaptations. The circadian clock in the SCN integrates variations in daylength, and thus encodes photoperiod. Then, it directly generates the rhythms in melatonin synthesis and secretion within the pineal. This synthesis occurs during the dark period of the light/dark cycle, the duration of melatonin secretion being directly dependant upon the duration of the dark phase. It is this change in duration of the nocturnal peak of melatonin synthesis/secretion that is the critical parameter in the integration of the photoperiodic information by the central nervous system. How the photoperiod throughout the melatonin rhythm controls all these seasonal functions remains undetermined. In hibernating rodents (or in rodents with daily torpors), the shortening daylength (SP) occurring in autumn induces a gonadal regression and a decrease in plasma levels of sex hormones. Since the gonadal hormones are known to act in the brain itself, it has been suggested that the timing of some other seasonal phenomena, such as hibernation/daily torpor, may result secondarily from the photoperiod-induced changes in gonadal hormones. The results obtained in the European hamster (*Cricetus cricetus*), the jerboa (*Jaculus orientalis*), the garden dormouse (*Eliomys quercinus*), and the Siberian hamster (*Phodopus sungorus*) confirm this interpretation. They can be summarized as follows: (1) the annual sexual cycle is synchronized with the season by the changes in photoperiod via the changes in the melatonin secretion patterns; (2)

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the reduction in testosterone plasma level subsequent to SP induces changes in central vasopressinergic (VP) innervation especially in the lateral septum (LS), a structure known to be involved in the control of temperature set point. Such changes allow the body temperature to fall below normal level when the ambient temperature is decreasing; (3) after several months of hibernation, the testes begin to increase in size (photorefractory phase) and the reactivation of testicular function might serve as a stimulus, via progressive reestablishment of VP innervation, to break of hibernation. The photoperiod thus controls two seasonal functions: one directly, the reproductive cycle, and the second one the hibernation (or daily torpor) indirectly via its effect on the reproductive cycle. However, a direct effect of the photoperiod on the hibernation has also been demonstrated. For example, in castrated hamsters kept under long photoperiod, there is also an absence of VP stained fibres in the LS, but these animals are unable to present hypothermic bouts. They need to be exposed to SP to express the hibernation cycle. Interestingly, the development of new pharmacological tools to study melatonin action has permitted us to demonstrate that the direct photoperiodic effect on hibernation involves other structures than those controlling the seasonal reproduction. The melatonin antagonist S20928, indeed, strongly decreases the number of hypothermic bouts (as well as the mean duration of the bout) without affecting the SP-induced gonadal atrophy.

Introduction

Life on the earth is exposed to strong rhythmic environmental changes caused by planetary movements. To ensure that certain events occur at optimal time-points each year (e.g., moult of skin, changes in fur and plumage colour, thermoregulation, hibernation, migration, reproduction, etc.), animals have to adapt to these variations in the environment. Changes in daylength (photoperiod), constant through the years, are used by animals to anticipate the forthcoming season. The mechanism used by mammals for this purpose is built around three key components: (1) the retinal photoreceptors that register and transmit environmental light cues; (2) a clock, located in the suprachiasmatic nucleus of the hypothalamus (SCN) that generates rhythms with a period of about 24 hours and is capable of being entrained to exactly 24 hours by the light/dark (LD) cycle. The molecular mechanisms that underlie the function of this biological clock consist of gene-protein-gene feedback loops in which proteins can downregulate their own transcription and stimulate the transcription of other clock proteins (Okamura et al., 2002). The phases between these interlocking

transcriptional/translational feedback loops are dependent on the length of day, demonstrating that the SCN also encodes photoperiod (Tournier et al., 2003). (3) Endocrine and neuroendocrine effectors receiving, via nervous and endocrine pathways, signals from the clock and translating them into hormonal or neurohormonal responses (Buijs and Kalsbeek, 2001). Among these effectors is the pineal gland, which secretes the hormone melatonin (Mel). Mel is synthesized and secreted during the dark period of the light/dark cycle, and the duration of the nocturnal production of melatonin is proportional to the length of the night. It has been demonstrated that photoperiodic information is integrated through these changes in duration of Mel synthesis/release (Bartness et al., 1993, Pévet 2002). The photoperiod is thus transduced into an endocrine message (nocturnal Mel secretion) and finally deciphered by melatonin target tissues that ultimately regulate overt behavioural and physiological changes. Studies on the role of photoperiod are mostly centred on its effect on the annual reproductive cycle. How this signal controls other seasonal functions remains undetermined. To understand these regulations, the sites and mechanisms of melatonin action should be considered first.

Melatonin

Mel exerts its effects principally through high affinity G-protein coupled receptors. Different types of melatonin receptors (MT1, MT2, MT3) have been cloned (Weaver 1999) and this is probably only the beginning of a long list (Pévet, 2002). Considering photoperiodic responses, the melatonin receptors involved are most probably of MT1 subtype (Weaver et al., 1996). The Mel target sites mediating the control of photoperiod-dependent seasonal functions, and especially the annual sexual cycle, have not been fully determined. In mammals, melatonin receptors are present in a large number of structures (more than 110 brain structures) (Masson-Pévet et al., 1994a). However, a great variability has been noted in the number and location of these structures among species. Furthermore, receptor density between these structures and in the same structures between species varies considerably. Only a few structures contain melatonin receptors in several species, even when considering related species (Masson-Pévet et al., 1994a). This probably reflects the numerous photoperiodic responses, which differ from one species to another. One structure, however, the *pars tuberalis* of the pituitary (PT) contains a very high density of Mel receptors in all mammals studied. The implication of the PT in the control of seasonal secretion of prolactin has been demonstrated (Morgan et al., 1996).

The PT is thus a good model to delineate the Mel signal transduction pathways and to study how the cellular response can distinguish between long and short duration Mel signals. The cAMP-mediated pathway appears to be central to the Mel readout. Mel has a duration-dependent effect on the sensitization of adenylate cyclase and potentiates the cyclic AMP response to forskolin stimulation (Hazlerigg et al., 2001). The nocturnal Mel signal is also crucial for the rhythmic expression of several cyclic AMP responsive genes (e.g., ICER) as well as of several clock genes in the PT. *Per1* and *Cry*, two components of the molecular clock, are rhythmically expressed in the PT (Messenger et al., 2001, Dardente et al., 2003). Furthermore, other components of the clock like *Timeless*, *Clock*, and *Per2* are also expressed in the PT. Clock gene expression in the PT seems to differ from that in the SCN or other peripheral tissues (peripheral oscillators) and appears to be directly driven by Mel. *Cry1* expression is acutely and transiently induced by Mel, providing thus a sensor for the onset of Mel secretion, rather than a marker of the duration of the melatonin signal. *Per1* expression, on the other hand, appears to be linked to the offset of melatonin secretion. This dual effect of Mel on *Cry1* and *Per1*, combined with its photoperiod-dependent pattern in plasma levels may provide the basis of a time measurement mechanism in the PT (Hazlerigg et al., 2001). However, a complete understanding of the melatonin and photoperiodic readout requires a link with an identified downstream response in the PT.

Photoperiod-induced changes in prolactin, however, cannot totally explain the seasonal changes in sexual activity. To mediate photoperiod, Mel must also act on other target sites. This is supported by the fact that Syrian hamsters bearing lesions in the dorsomedial hypothalamus (DMH) and infused with Mel display differential responses to photoperiod for prolactin and luteinizing hormone (LH). While the prolactin response remains intact, the LH response is blocked by the DMH lesion (Maywood and Hastings, 1995). Moreover, in the sheep, Mel implants in the mediobasal hypothalamus block the effects of SP on LH but not on prolactin, while implants close to the PT inhibit prolactin secretion (Malpoux et al., 2001).

Mel thus acts at multiple sites to induce an integrated set of physiological responses adapted to the seasonal changes of the environment. The parallel and concomitant action of Mel on different structures is probably species-dependent in order to control the photoperiod-dependent seasonal functions relevant to a particular species. Indeed, not all seasonal functions are expressed in every species and different control mechanisms exist. Short photoperiod induces an

activation of the sexual axis in the sheep yet inhibition in Syrian and Siberian hamsters. Hibernation in the Syrian hamster is directly dependent on the photoperiod while, in the European hamster, it is dependent on a circannual clock entrained by photoperiod. This hypothesis thus may account for the large interspecies differences observed in mammals in the distribution of Mel receptors. Interestingly, S22153, a Mel receptor antagonist, caused a decrease in the duration of hibernation in Syrian hamsters under short photoperiod and low temperature, but did not affect SP-induced gonadal atrophy (Pitrosky et al., 2003). This pharmacological dissociation of photoperiod-controlled seasonal functions supports the model of the species- and function-dependent multisite action of melatonin.

Reality, however, is still more complex than this model. In numerous species, seasonal changes of reproductive activities are directly involved in the regulation of other seasonal functions. A low level of testosterone, for example, is a prerequisite for hibernation or daily torpor in rodents. Since the gonadal steroids cross the blood–brain barrier to bind specifically to some brain structures and to influence centrally controlled functions, it has been suggested that the timing of some seasonal phenomena such as hibernation/daily torpor may result secondarily from photoperiod-induced changes in gonadal activity.

Gonadal Steroids as Mediator of the Photoperiodic Message

The Hibernation Cycle: Relationship to Photoperiod and the Gonadal Activity

The physiological mechanisms triggering hibernation are far from being completely understood. In the present review we will not consider the phenomenon of hibernation proper, but the annual cycle of hibernation and its synchronization by photoperiod.

In the male European hamster kept under natural conditions of photoperiod and temperature, hibernation starts in autumn and lasts until the beginning of spring. The transition from euthermia to a regulated hypothermia is only possible if the hamster has undergone a period of physiological preparation, especially an arrest of gonadal activity, in summer and early autumn. Reproduction in the European hamster is regulated by photoperiod (Masson-Pévet et al., 1987). Gonadal regression occurs in August when day length decreases. Gonadal activity is spontaneously reactivated (photorefractory phase) in March–April, when animals still hibernate in the dark in their burrow (Canguilhem et al., 1988a,b). In the European hamster, like in numerous other hibernating species (e.g., the Turkish hamster, golden hamster, hedgehog, jerboa, and garden

dormouse), the hibernation cycle shows an inverse phase relationship with the gonadal cycle. Hibernation starts when gonadal activity is at its minimum and stops in April when the process of endogenous reactivation of gonadal activity is finished. It has also been demonstrated that both processes are mutually exclusive. Implantation of testosterone-filled silastic capsules at the end of July, just before the start of gonadal involution induced by the natural decrease in day length, completely prevents hibernation. As a matter of fact, experimental data obtained in most hibernators show that a low level of testosterone is a prerequisite for the expression of hibernation. This explains most of the described effects of the photoperiod on the hibernation cycle. European hamsters captured in the field in April at the end of the hibernation period and kept then under a continuous short-photoperiod (SP) are unable to hibernate when placed outdoors in October under natural conditions or in SP at 6° C. However, control animals that remained throughout spring and summer either in a long photoperiod (LP) or outdoors under a natural LP hibernate normally (Canguilhem et al., 1988a,b). It has been established that the animals are in a photorefractory state at the end of the hibernation period. If this state is not abolished by exposure to LP, the animals mostly remain continuously sexually active. Thus the effect of the photoperiod on hibernation appears to be an indirect one, consecutive to the changes in gonadal activity induced by photoperiod treatment.

However, some observations, e.g., in the European hamster, seem to contradict this conclusion. Even when kept under a long photoperiod at 20° C throughout summer and autumn, a condition that normally prevents short photoperiod-induced gonadal atrophy, some European hamsters still displayed some periods of hypothermia in autumn. The animals that showed a period of hypothermia were always sexually inactive, while sexually active ones failed to hibernate. This observation can be explained by the existence of an annual "clock" in this species, which controls the sexual cycle and which is synchronized to one year by changes in the photoperiod (Masson-Pévet et al., 1994b).

The temporal physiological relationships between the annual gonadal and hibernation cycles in hibernating rodents implies that gonadal hormones control the activity of neuronal systems involved in the hibernation process. We will concentrate on the vasopressin systems, for which numerous data have already been obtained.

Vasopressin Systems in the Brain: Relationship with the Gonadal Cycle and Hibernation

Vasopressin (VP) is released from neurons residing in the supraoptic (SON) and paraventricular nucleus (PVN) that projected to the posterior pituitary. However, VP is not only present in the classical hypothalamo-neurohypophyseal system, but also in an extensive nerve fibre system throughout the brain (Kalsbeek et al., 2002). These fibres originate from neurons situated in several nuclei such as the PVN, the SCN, the bed nucleus of the stria terminalis (BST), and the medial amygdala (Kalsbeek et al., 2002). VP thus also acts as a neurotransmitter or a neuromodulator and may be implicated in several centrally regulated functions.

Furthermore, central VP systems are also known to interact with gonadal steroids in a variety of ways. For example, the amount of VP in fibres originating from the BST is dependent on the levels of circulating testosterone. Even if other neuropeptide systems show variations in their expression in response to gonadal steroids (Lakhdar-Ghazal et al., 1995a; Oukouchoud et al., 2003), the effects on the VP system in the BST are among the most dramatic reported. Following castration of male rats, jerboas, and European or Djungarian hamsters, VP-fibre density in the lateral septum (LS), the lateral habenular nucleus, and other projection areas of the BST (De Vries et al., 1986) gradually decreases until virtually no VP-immunoreactive fibres remain (Buijs et al., 1986; Dubois-Dauphin et al., 1994). Similar changes are observed under naturally decreased gonadal activity, i.e., during aging (Fliers et al., 1985) or during the nonbreeding season in seasonal species (Buijs et al., 1986; Hermes et al., 1990; Lakhdar-Ghazal et al., 1995a; Ouarour et al., 1991). VP mRNA levels also decline, indicating that testosterone controls VP at the transcriptional level (Szot and Dorsa, 1994).

Moreover, in sexually quiescent European hamsters, Djungarian hamsters, or jerboas (October–November) VP-ir cells and fibres reappeared after testosterone replacement. This close relationship between the circulating testosterone levels and the VP innervation also explains the results obtained after manipulation of either the photoperiodic regime or Mel levels (Pévet et al., 1987).

Hibernation is characterized by regularly occurring periods of hypothermia. VP in the lateral septum is involved in the maintenance of a normal body temperature. In the European hamster as well as in the jerboa or other hibernators, a period of sexual inhibition of one to two months is necessary to start hibernation. This is also the period necessary to obtain a complete disappearance of the VP innervation in the lateral septum after either castration or SP exposure (Buijs

et al., 1986). Moreover, testosterone implants in autumn and winter prevent the disappearance of the VP innervations in the lateral septum and inhibit hibernation. Similar results have been obtained in the Djungarian hamster (Ouarour et al., 1991, 1995). The disappearance of VP fibres from the lateral septum in autumn-winter thus appears to be necessary for the expression of hibernation (or daily torpor). An experimentally induced persistent VP release in the lateral septum disturbed the hamster's ability to hibernate. The appearance of bouts of hypothermia in most of the VP-infused animals directly after the end of VP release indicated that at that moment VP infusion was the only factor preventing hibernation (Hermes et al., 1989).

These results demonstrate that the testosterone-dependent VP system plays a critical role in the physiological mechanism enabling the photoperiod to time the hibernation cycle. They also explain, at least partially and in the few rodents expressing hibernation or daily torpor cycles studied to date, how the photoperiod can time two seasonal functions. This mechanism can be summarized as follows (see Pévet et al., 1987, 1990):

1. Via a partially understood mechanism involving the circadian clock, the pineal and its hormone, melatonin, the annual photoperiodic changes synchronize the sexual cycle with the seasons.
2. The reduction of testosterone levels subsequent to short daylight induces changes in the central VP innervation in some brain areas, especially a disappearance of VP in the lateral septum. When the ambient temperature decreases, this absence of VP in the LS allows the body temperature to fall below the normal level.
3. After several months of hibernation, the photorefractory period starts and circulating testosterone levels increase. This increase stops, via among others a progressive reestablishment of the VP innervation in the LS, the period of hibernation or of daily torpor.

Nongonadal Mediated Effects of the Photoperiod on the Hibernation Cycle

A direct effect of the photoperiod on the hibernation cycle, independent of its indirect effect via modification of gonadal activity, has also been demonstrated. In castrated male European hamsters the hibernation cycle proceeds normally, terminating after several months. Thus, although delays in break-off of hibernation were noted, under these experimental conditions testosterone is not essential for the termination of hibernation. A similar conclusion also emerged from experiments by Goldman and Darrow (1987) using another hibernator, the Turkish

hamster. Is the photoperiod implicated in this termination of the hibernation cycle in castrated animals? European hamsters caught at the end of the hibernation period (photorefractory phase) and immediately castrated were kept under a continuous SP, while castrated control animals were exposed to a normal LP during spring and summertime. In October, the animals were transferred either outdoors or under SP at $6^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The castrated hamsters maintained under SP did not hibernate, while the castrated controls that were exposed to LP did (Canguilhem et al., 1988a, 1988b). Under these conditions testosterone cannot be considered as responsible for the nonexpression of the hibernation cycle. It appears that the European hamster, in order to hibernate, not only needs to decrease its sexual activity but also needs to break off the photorefractory period by exposure to LP in spring and summertime. Which are the physiological mechanisms implicated in this aspect of the photoperiod response? The photoperiod probably acts at the level of one (or more) of the brain Mel target structures, but this still remains to be clearly established. Also, these melatonin target structures have not yet been identified. However, the experimental dissociation of photoperiod-controlled seasonal functions (hibernation and reproduction) obtained with a Mel receptor antagonist supports this model (Pitrosky et al., 2003).

Conclusion

Although the role of the photoperiod in the control of seasonal functions and especially hibernation and reproduction has been recognized, our present knowledge of the exact neural and neuroendocrine systems involved in the timing of these functions is still poor. The present mini-review points to the importance of gonadal steroids and of the central vasopressinergic innervation. However, the BST-vasopressin system is not the only neurotransmitter system implicated. Other systems are also involved (Menet et al., 2003; Moller et al., 1998; Lakhdar-Ghazal et al., 1995b), but their exact implication needs to be evaluated experimentally.

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Behaviour, Body Temperature, and Hibernation in Tasmanian Echidnas (*Tachyglossus aculeatus*)

STEWART NICOL, CHRISTINA VEDEL-SMITH, AND NIELS A. ANDERSEN
University of Tasmania, Anatomy and Physiology, Hobart, Tasmania, Australia

Abstract. We investigated the interrelations between activity, thermoregulation, reproduction, and hibernation of free-ranging echidnas by using data loggers to record activity and T_b . This has helped us further define the pattern of hibernation of Tasmanian echidnas: males entered hibernation in late summer with the final arousal in winter; females entered hibernation in autumn, with the final arousal from hibernation in winter if they were to reproduce, or in spring if they did not reproduce that year. The activity loggers revealed an unexpected degree of nocturnality and showed the large daily T_b cycle could be largely attributed to activity.

Introduction

Echidnas have long been characterised as having low and labile body temperatures (T_b), even when not hibernating. Wardlaw (1915) measured T_b of captive echidnas twice daily using a rectal thermometer and found a “fairly constant” T_b (30–33° C), although there was “a daily variation which seemed to be, to some extent, independent of the variations of the external temperature.” This early investigator also noted that external temperature did have some effect since T_b was higher in spring than in autumn. Most recently, Brice et al. (2002) have investigated the relationship between the daily cycles of T_b and activity in free-ranging echidnas, by observing animals with implanted temperature transmitters, and found T_b rises to be associated with periods of activity. Rismiller and McKelvey (1996) radiotracked echidnas with implanted temperature transmitters for consecutive days to determine seasonal patterns of movements in the field and their relationship to hibernation and torpor. They found a general trend towards

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decreased movement prior to courtship at the beginning of June, and at the beginning of August after the breeding period, although inactivity did not necessarily indicate torpor.

Both of these studies have relied on fairly gross measurements of activity and were restricted to occasional sampling with nighttime observations being severely limited.

Since the first recording of body temperatures of hibernating echidnas in the field (Grigg et al., 1989), a number of investigators have remarked on the similarity of patterns to those in eutherian hibernators (Grigg and Beard, 2000; Nicol and Andersen, 1993; Nicol and Andersen, 2000; Rismiller and McKelvey, 1996). Before entry into hibernation proper, the normal circadian variation in T_b becomes exaggerated, with the daily minimum progressively falling. T_b during torpor then falls to within about 0.5°C of ground temperature and falls progressively during the hibernation period as surrounding temperatures fall. Just as in eutherian hibernators, continuous torpor is broken by periodic short arousal episodes. However, as has been noted previously (Nicol and Andersen, 1996; Nicol and Andersen, 2002), echidnas enter hibernation earlier and emerge much earlier than eutherian hibernators. An additional unique feature is that female echidnas switch between two strategies: in years in which they are not reproductively active they hibernate until spring but in years in which they reproduce they arouse nearly two months earlier (Nicol and Andersen, 1996; Nicol and Andersen, 2002).

To obtain more information on the thermal physiology of echidnas and on the relationship between reproduction, activity, and hibernation, we fitted echidnas with data loggers to record body temperature and activity. Additional information on behaviour was obtained by observing the animals in the field when possible.

Methods

The study was carried out at our field site at Lovely Banks, 50 km north of Hobart (Nicol and Andersen, 2000). Echidnas were captured by hand and implanted with passive transponder (PIT) tags. Twelve echidnas (eight female, four male) were implanted with data loggers (Onset Computer Corporation, Stowaway Tidbit) to record T_b and a tracking transmitter (Biotelemetry Tracking) glued to the spines of the lower back. After this they were located by radio tracking and their location recorded using a GPS receiver. Five echidnas (three female, two male), three of which had previously been implanted with T_b loggers, were fitted with activity loggers (Minimitter Actiwatch). A metal

backing plate was glued to the spines on the lower back opposite to the transmitter, and the logger unit, which also incorporated a second tracking transmitter, was fastened to the backing plate by screws, allowing it to be easily removed in the field.

Air temperature (T_a) was measured at one point in the field site by attaching a temperature logger to a tree trunk so that it was in the shade and 1.5 m above the ground. All values are given as means \pm SD unless otherwise noted.

Results

Body Temperature

Fig. 1 shows a three year recording of T_b from a female echidna, along with T_a and illustrates a number of characteristic features, including a low and variable

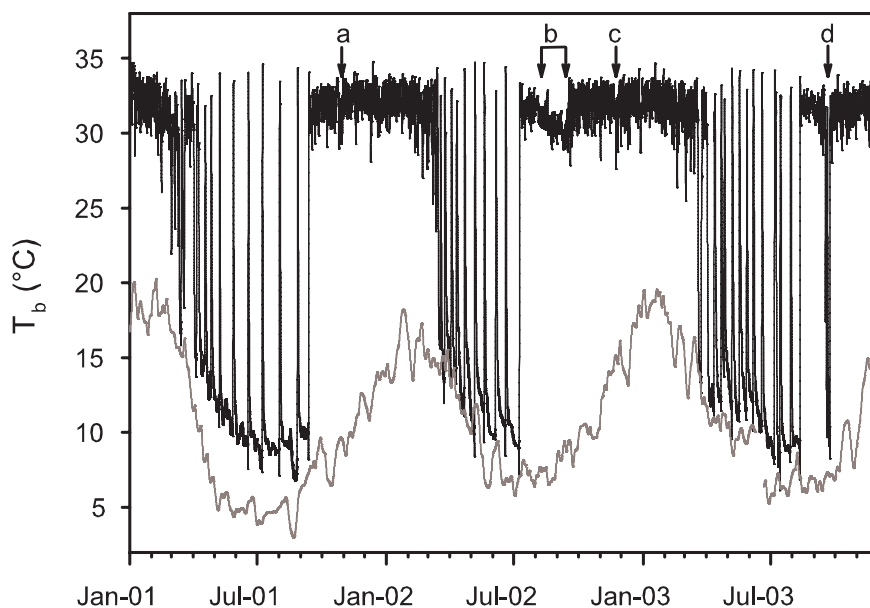


Fig. 1. T_b of a free-ranging female echidna over three years and smoothed average air temperature (grey line), showing a long bout of hibernation in a nonreproductive year (2001) and shorter bouts in reproductive years (2002 and 2003). Arrows a and c show the reduction in T_b variability associated with being held in captivity while external tracking transmitter was replaced, b shows the characteristic reduction in T_b variability in the period in which the echidna spent much of her time in the nursery burrow, and d a brief reentry into hibernation after probably losing an egg.

T_b which is highest in summer, an increase in variability before entry into hibernation proper, a progressive decline in minimum T_b as ground temperature falls, and frequent arousals. The mean active T_b for the data shown in Fig. 1 is 31.6°C , with a mean daily range of $3.1 \pm 1.0^\circ\text{C}$ ($30.1\text{--}33.2^\circ\text{C}$, $n = 680$ days).

We have now collected 35 echidna years of such T_b data from the eight female and four male echidnas, and Fig. 2 summarises information on entry into hibernation and final arousal based on all T_b data from undisturbed animals at our field site. Some entry data have not been included as late implantation of temperature loggers delayed entry into hibernation (Nicol and Andersen, 2002). In years in which they were not reproductively active, female echidnas hibernated until spring (entry March 12 ± 20 days, $n = 12$; arousal Sept 25 ± 15 , $n = 18$) but in years in which they reproduced they aroused nearly two months earlier ($t = 7.4$, $P < 0.0001$) in mid-winter (entry March 15 ± 13 , $n = 5$; arousal July 30 ± 20 , $n = 9$). The time of entry for males (February 15 ± 13 , $n = 6$) was significantly earlier than females ($t = 3.35$, $P = 0.003$), and, as in females, arousal dates seemed bimodal (June 7 ± 14 , $n = 5$, and August 16 ± 9 , $n = 3$), but males in both groups were reproductively active.

Activity

Activity loggers were deployed on five echidnas (three females, two males) for periods ranging from 6 to 180 days. On seven occasions the loggers were found to have been affected by water or had been physically damaged. A total of 550 days of activity were recorded, including 208 days of nearly continuous recording from one female. Fig. 3 shows a part of an actogram for one echidna (male 1E7C). The first part of the graph demonstrates an unexpected observation: some echidnas showed a high degree of nocturnality. Halfway through this record the echidna entered torpor, and the small bursts of activity are associated with the

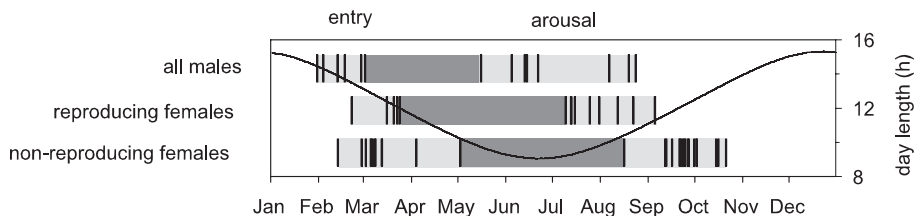


Fig. 2. Summary of the timing of hibernation and entries and emergence. Individual dates are indicated by vertical lines. Dark shading indicates period between last entry and first arousal for that group. Curve shows day length.

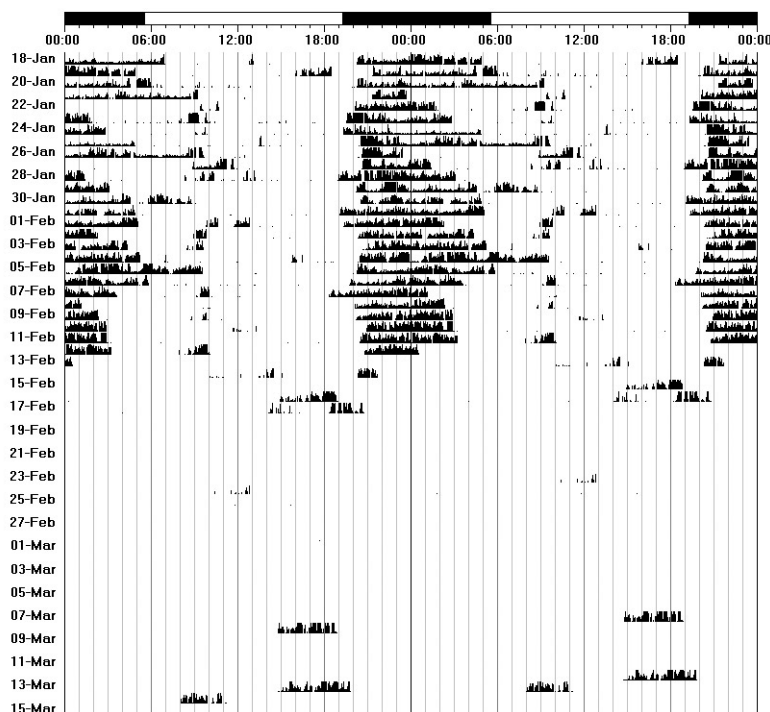


Fig. 3. Two-month record of activity from a male echidna (1E7C) in 2003. Data are displayed in dual day format, so each point appears twice. Activity is logged over two-minute epochs.

periodic arousals. In some cases animals would relocate during periodic arousals, which could involve moving from one side of the home range to the other.

Discussion

The Relationship Between T_b , T_a , and Activity

Fig. 4 shows the average daily cycle of T_b in male echidna 1E7C over the period from the summer solstice until one week before entry into hibernation. Also shown on the figure is the hourly average activity over the same period, clearly demonstrating the very close correlation between T_b and activity. Fig. 5 shows activity, T_b , and air temperature during entry into hibernation and the six days preceding entry, showing again the close correlation between activity and T_b . In this case T_b and activity are out of phase with T_a . Figs. 4 and 5 strongly suggest that

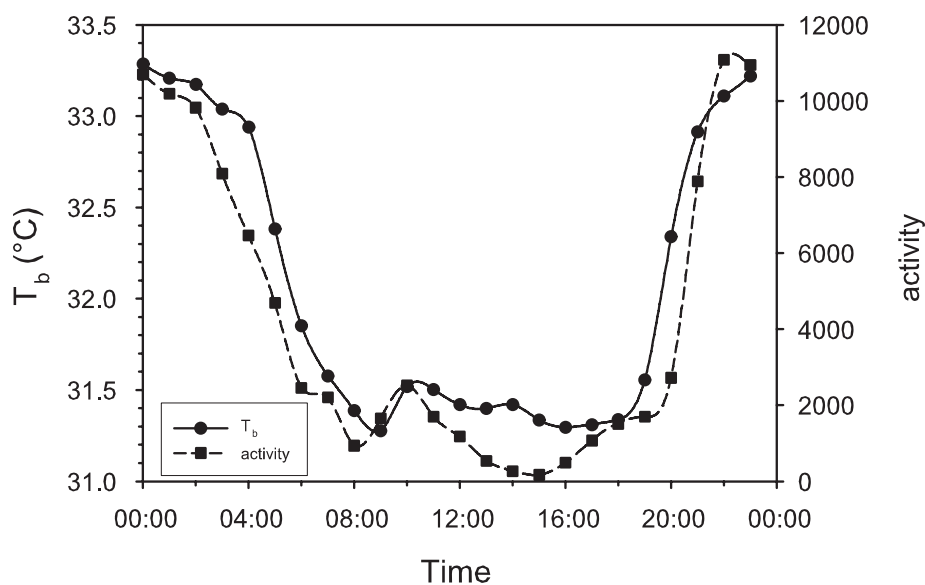


Fig. 4. Hourly average T_b (solid lines) and activity (broken lines) for male echidna 1E7C over the period 22 December–6 February.

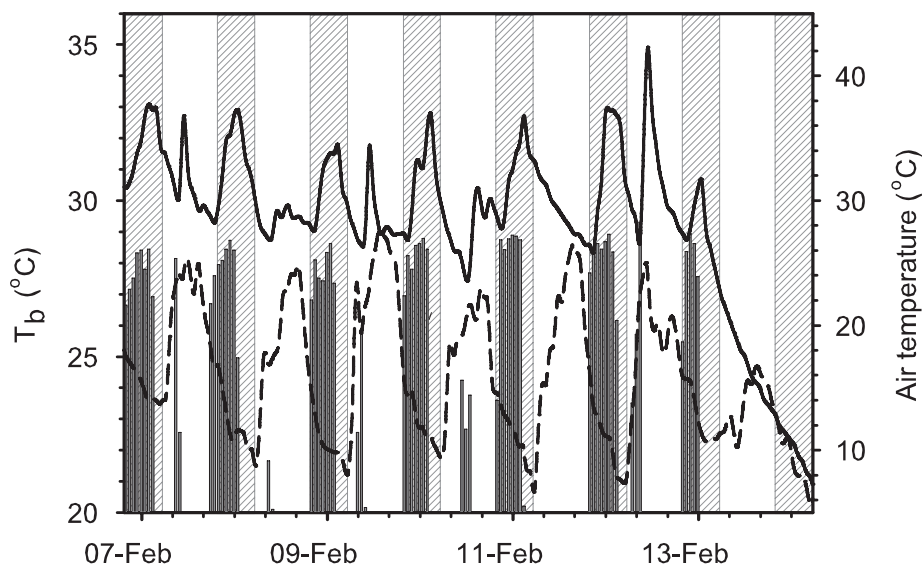


Fig. 5. T_b (solid lines), T_a (broken lines) and activity (bars) for male echidna 1E7C immediately before entering hibernation. Shading shows dark period.

the characteristic large diurnal variation in T_b of echidnas is a simple function of the activity cycle and that departures from this daily T_b cycle are associated with a change in the activity pattern. As noted above the activity records of some echidnas showed an unexpected degree of nocturnality, and we are in the process of making a detailed analysis of all activity data (Vedel-Smith et al., in prep). The effect of activity on T_b is illustrated in Fig. 1 where short periods of reduced T_b variation in late October 2001 (*a*) and late November 2002 (*b*) occurred when the animal was briefly held in captivity for transmitter replacement. It has been noted previously (Beard et al., 1992; Nicol and Andersen, 2002) that during the period when the female is carrying an egg, and in the first few weeks following hatching, there is a period of reduced T_b variability. At this stage most females in the colder parts of Australia spend their time in a nursery burrow, and there is probably a reduction in overall activity. Although we were not able to observe the female shown in Fig. 1 during this time, the T_b record at *b* in July–August 2002 shows the characteristic T_b changes of a female that has hatched an egg.

Fig. 1 supports the suggestion of Wardlaw (1915) that season affects the baseline T_b in active animals, but in fact maximal T_b occurs during early, rather than late summer, i.e., the highest T_b occurs before the highest T_a , and probably reflects, in part at least, the high levels of feeding activity at this time. More activity data and more detailed analysis are required to confirm this. During hibernation minimum T_b tracks T_a , and arousals may be triggered by warm spells.

Hibernation and Reproduction

As appears to be the case in all hibernators other than bats (Wimsatt, 1969), reproductive activity of echidnas follows closely on arousal from hibernation, and the entire reproductive sequence from insemination to the weaning of young must be completed before the next reproductive cycle. On average, the females in this study reproduced in one out of three years, but we did select females that we thought were likely to breed (see below). However there was great variation between females, with one individual breeding in two out of every three years. Rismiller and McKelvey (2000) found a similar very large variation between female echidnas on Kangaroo Island, with an overall breeding success of one young in four years. The closest comparison amongst other hibernators seems to be the hoary marmot (*Marmota caligata*). Females of this species normally breed biennially, but may miss two consecutive seasons (Wasser and Barash, 1983). In this and other marmot species, failure to breed is the result of suppression by more dominant females in the colony (Oli and Armitage, 2003; Wasser and

Barash, 1983). We do not have enough data to demonstrate if the failure by some female echidnas to breed in some years is a response to weather conditions as has been documented in bats (Lewis, 1993), but failure by some female echidnas to breed may not be reproductive failure *sensu* Wasser and Barash (1983) and may be determined before the echidna enters hibernation, with the female deciding to hibernate through the breeding season. Although females seem more likely to breed when they are heavier, the relationship is not clear cut, and it is possible that the decision to breed may also be affected by conditions during one of the periodic arousals, which become increasingly frequent and irregular in the second stage of hibernation (Nicol and Andersen, 2000).

Because we selected large females for this study in the belief that they were more likely to arouse early and mate, we mainly have T_b recordings from large females. The smallest female included in this study was 2.8 kg when found in 1997 and was then classified as a young female. Fig. 6 shows her T_b record for 2000 and shows that her final arousal from hibernation was on August 15, 40 days earlier than her previous earliest arousal date. After several weeks of activity she then reverted to bouts of torpor for the next month. We believe this represents a first, unsuccessful attempt to mate, and we observed the first successful

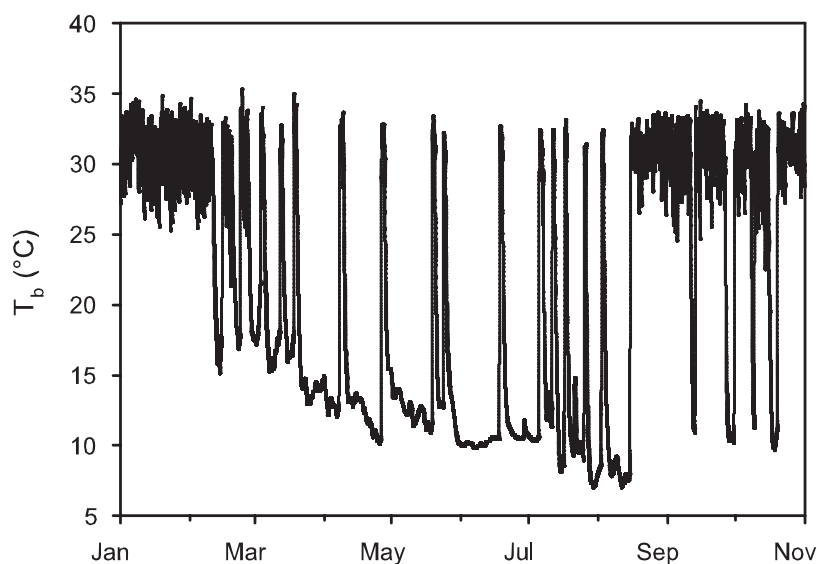


Fig. 6. T_b record of a female echidna showing an early arousal and reentry into hibernation after what appears to be an unsuccessful first mating season.

mating in 2002 when she weighed 3 kg. Echidnas are solitary, but pheromones appear to be very important in attracting males to females (Rismiller and McKelvey, 2000), and we have also noted that both sexes have a pungent odour during mating. Hence some females may be influenced by pheromonal signals from more dominant females or by an absence of male pheromones. Although we do not yet know what the proximate causes are when females hibernate rather than reproduce, it is likely to be an energy-saving adaptation to the relatively unpredictable Australian climate that could only be exploited by such a long-lived species (Nicol and Andersen, 2002). A further energetic advantage is provided by the short gestation period (22–24 days, Rismiller and McKelvey, 2000), and the very small size of the young at hatching (0.3 g) which mean that relatively little energy will have been invested in young lost in the first month or so (Nicol and Andersen, 2002), and the energy lost by not hibernating is greater than that invested in a young. We have shown previously that field metabolic rate of lactating females is not higher than nonlactating females at the same time, indicating a reduction in other metabolically expensive processes, such as activity and thermoregulation (Schmid et al., 2003).

As has been noted previously, hibernation in echidnas is not a response to extreme cold or food shortages (Nicol and Andersen, 1996). The latest entry into hibernation was May 2, 2003, for a nonreproductive female that then hibernated for 167 days, while the earliest arousal was 16 May 2001 for a male that had hibernated for 106 days. Although all echidnas in our study hibernated, the periods of hibernation for the different groups overlap only slightly (Fig. 2). Males often entered hibernation at the warmest time of the year, and they emerged before the winter solstice. These patterns appear to result from a drive to maximise the period of hibernation while ensuring matching of male and female reproductive cycles, since males may require several weeks of euthermic temperatures before spermatogenesis can occur, as has been demonstrated in ground squirrels (Barnes, 1996). More information is required on reproductive activity to help understand the energetic consequences of this behaviour.

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Metabolic Diversity in Yellow-Bellied Marmots

KENNETH B. ARMITAGE

University of Kansas, Ecology and Evolutionary Biology, Lawrence, Kansas, USA

Abstract. Yellow-bellied marmots conserve energy. Oxygen consumption of young, yearlings, and adults declines linearly after the midsummer molt, and field metabolic rates (FMR) are significantly lower than predicted from body size. Metabolism increases at an environmental temperature below measured burrow temperatures, which contrasts with results from laboratory-held marmots where metabolism increased at burrow temperatures. Metabolism of young and adult females increased in a drought year in comparison to normal years. Metabolism of adult males peaked in early summer when conflict among males is common. FMR of females declined linearly throughout the summer. FMR of reproductive females was maximal during lactation but did not differ from that of social-conflict males. Costs of reproduction may be higher for males because males, engaged in territorial conflict, maintain high FMR about twice as long as reproductive females. Marmots apparently maintain minimal metabolism and increase it for special functions such as lactation and territorial conflict.

Introduction

Most species of marmots live in harsh environments characterized by stormy, cold environments in a landscape that may be snow covered until July. Under these conditions reproductive skipping typically occurs, reproduction may begin in the burrow before emergence, embryo reabsorption may occur, and mass loss may continue for several weeks after emergence (Armitage and Blumstein, 2002). These conditions suggest that marmots that reduce energy expenditures and increase energy for accumulating fat for hibernation and post-hibernation activities would have a major fitness advantage.

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

Considerable evidence, such as rates of metabolism that are lower than that predicted from body mass and a growth efficiency much higher than that of typical homeotherms, indicates that yellow-bellied marmots (*Marmota flaviventris*) are energy conservers (Armitage, 1998). This paper explores diverse patterns of energy use in yellow-bellied marmots in the Upper East River Valley, Colorado.

Materials and Methods

Oxygen consumption was measured for young, yearlings, and adults of both sexes during the active seasons of 1990 through 1995 at the Rocky Mountain Biological Laboratory at 18°–20° C, which lies in the thermoneutral zone of yellow-bellied marmots (Armitage et al., 1990), except for a series run at different ambient temperatures. When oxygen consumption was measured at different temperatures, marmots had at least one hour to adjust to the new temperature. Data were recorded when oxygen consumption became stable for at least 30 minutes. Reproductive state was determined by nipple condition (Armitage and Wynne-Edwards, 2002) and scrotal development.

Marmots were live-trapped in the morning, transported to the laboratory, and returned to their site of capture the same day. The few marmots that were held overnight during runs at different ambient temperatures were fed fresh native food plants in late afternoon, and runs occurred the next morning after which the marmots were released.

Because wide-ranging values occurred in some runs, we opened the growth chamber door to observe activity of selected marmots in the metabolism chamber to determine whether marmots were active or quiescent and the degree to which activity affected metabolism. For example, stable values were obtained when a marmot actively attempted to escape from the metabolism chamber, but these values were much higher than those of a quiescent (lying or sitting in the metabolism chamber, eyes open) or sleeping (lying with eyes closed) animal. Specific VO_2 ($\text{ml O}_2 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) increased from 175.8 to 304.4 during alarm calling, which could be heard in the laboratory, and VO_2 of seven yearlings was ($\bar{x} \pm \text{SE}$) 202 ± 16.0 while quiescent and increased by 65.9% to 335.1 ± 27.1 when active. We used these active values as indicators that marmots were not quiescent and runs with similar high values were not used in subsequent analyses. Thus, the reported values of VO_2 represent least observed metabolic rate (Blaxter, 1989:122) and probably approached resting metabolism.

Field metabolic rates (FMR) were measured by the doubly labeled water method from 1989 through 1993. All analyses were performed at the Boston

University Stable Isotope Laboratory. Methods of trapping and handling the marmots and for measuring oxygen consumption and procedures for determining FMR are presented in great detail elsewhere (Armitage and Salsbury, 1992; Salsbury and Armitage, 1994; Armitage and Woods, 2003). For FMR, we focused on marmots with a high probability of recapture, but recaptures were not always successful and sample sizes for reproductive females are small and unavailable for adult males after mid season. Predicted FMR was calculated as: $L\text{ CO}_2/\text{day} = 0.141\text{g}^{0.81}$. All data were analyzed by ANCOVA, ANOVA, or simple and multiple linear regression in MINITAB. In a few instances, differences between two means were tested by a T-test. Dates are recorded as Julian date, unless otherwise noted.

Results and Discussion

Effects of Environmental Temperatures

Laboratory marmots increased VO_2 35.6% from 15° C to 10° C (Armitage et al., 1990). This increase represents a considerable metabolic cost that was inconsistent with mean summer burrow temperatures that ranged from 9.0° C in June to 11.3° C in late August (Kilgore and Armitage, 1978), especially given that marmots spend nearly 18 hours each day in their burrows (Armitage et al., 1996). We hypothesized that the laboratory animals had acclimated to higher temperatures and that field animals would be acclimated to lower environmental temperatures. The hypothesis was supported; when adjusted for the significant effect of body mass ($p = 0.0001$), temperature significantly affected both VO_2 ($\text{ml O}_2 \cdot \text{hr}^{-1}$) and specific VO_2 ($p = 0.0001$). For adults only, VO_2 at 5° C differed significantly from the other values (Fig. 1). VO_2 of males was significantly greater than that of females ($p = 0.0001$), but there was no temperature: sex interaction, and sexes were combined in Fig. 1. Body mass significantly ($p = 0.0001$) affected VO_2 but not specific VO_2 ($p = 0.13$) of yearlings. When adjusted for body mass, VO_2 ($p = 0.027$) and specific VO_2 ($p = 0.015$) of yearlings were significantly affected by temperature (Fig. 1); VO_2 at 5° C was significantly greater ($p < 0.05$) than VO_2 at the higher temperatures.

Seasonal Changes

Because VO_2 decreased markedly after the summer molt (Armitage and Salsbury, 1993), all measurements were made post-molt except for adult males. Molting was easily observed by the pattern of growth of new fur (Armitage, 2003). The molt begins on the hindquarters and proceeds anteriorly (Davis, 1966). Molt is

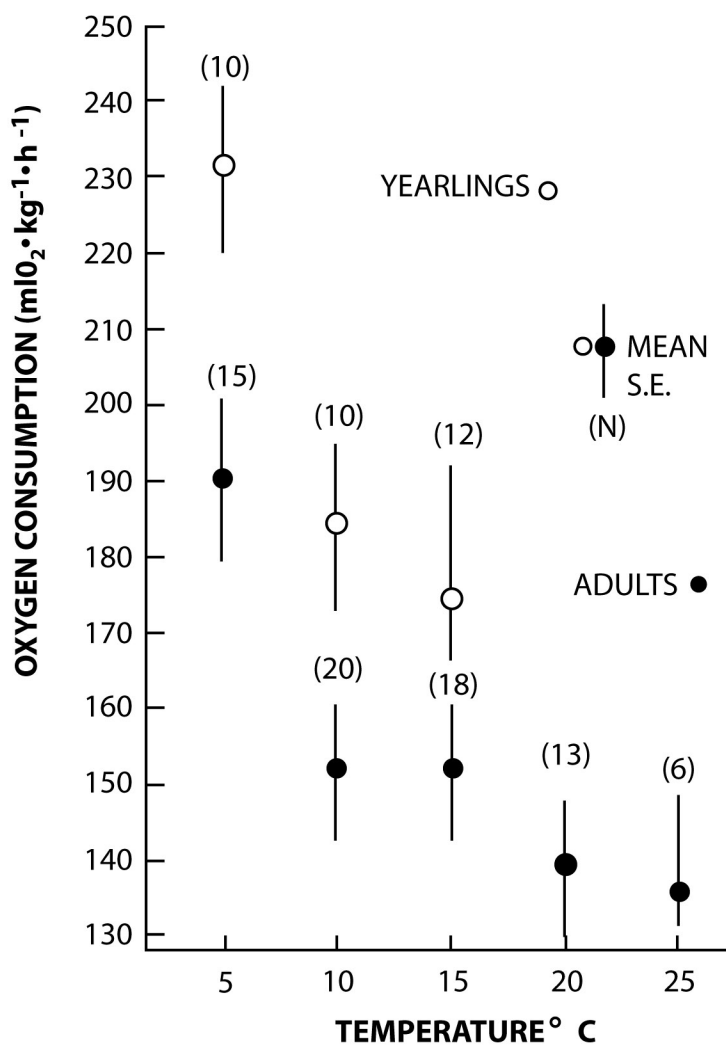


Fig. 1. The effect of environmental temperature on metabolic rate. For adults, body mass varied from 2.05 to 4.76 Kg; for yearlings, from 1.5 to 3.7 Kg.

delayed in reproductive females and occurs later at higher elevations (Bibikow, 1996). Therefore, all VO_2 values were standardized to time since molt was completed. Yellow-bellied marmots typically gain mass during the summer active season (Salsbury and Armitage, 2003); in this study mass increased significantly for young ($p = 0.0001$) and yearlings ($p = 0.06$), but not for adult females ($p = 0.13$) and males (0.8). VO_2 ($\text{ml}\cdot\text{h}^{-1}$) and specific VO_2 ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) of all groups declined seasonally. For young, mean specific VO_2 declined from 250.5 on day 220 to 135.6 on day 270. For yearlings, mean specific VO_2 declined from 268.0 on day 193 to 151.4 on day 248. For adult females, specific VO_2 decreased 21.4% from a mean of 226.2 during molting to the mean value eight days later of 177.8 and declined slowly thereafter to a mean of 109.4, 51 days after the completion of molting (Fig. 2). Thus, the post-molt decline is not just a consequence of molting but continues at least until the first week of September. Similarly, both Arctic ground squirrels (*Spermophilus parryii*) and yellow-bellied marmots had

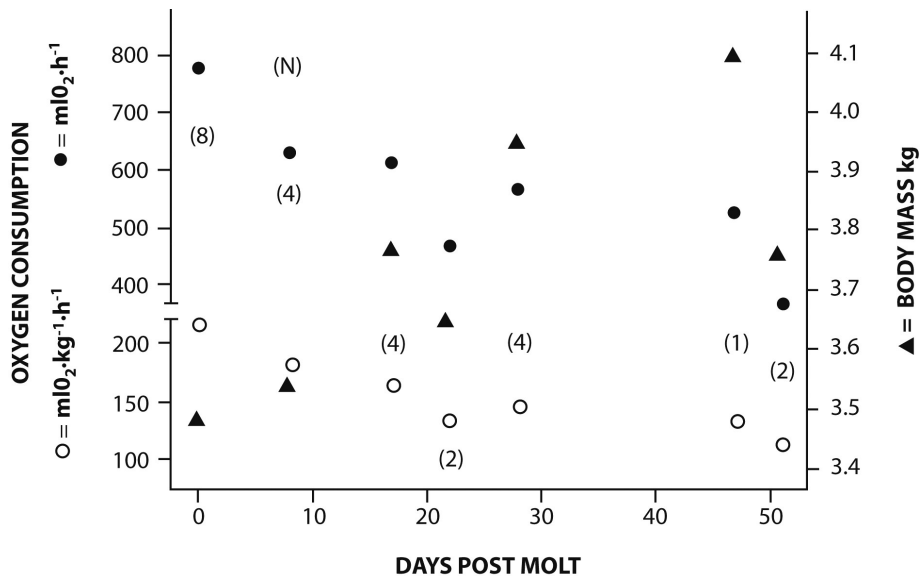


Fig. 2. The relationship between mean metabolic rate, mean specific metabolic rate, and mean body mass and the number of days post molt for adult females. Data from 1992 and 1993 combined. Regressions for this figure: Specific $\text{VO}_2 = 215 - 2.25 D$, $p = 0.018$, $R^2 = 0.74$; $\text{Kg} = 3.5 + 0.009 D$, $p = 0.042$, $R^2 = 0.6$; $\text{VO}_2 = 709 - 5.77 D$, $p = 0.017$, $R^2 = 0.71$. (N) number of females. Zero days refers to the molting period.

decreased VO_2 from August to September and high values the following July (Hock, 1969). Metabolic rate of a normothermic *M. marmota* had high values in spring and mid summer and low values in fall and winter (Ortmann and Heldmaier, 1992).

In the multiple regressions, the partial p for day was highly significant for all animal groups (Table 1) and the partial p for body mass was usually significant. The significant effect of day indicates that the seasonal decline in metabolism is not a consequence of the gain in mass of fat, a low metabolic rate tissue. For adult nonreproductive females, both VO_2 and specific VO_2 decline even though mass increases (Fig. 2).

VO_2 of several adult females declined seasonally, thus confirming that the decrease in VO_2 was not a consequence of variability among females but characterized all females. VO_2 of female 478 declined seasonally in 1993, but was unusually low in mid June 1995 (Fig. 3). The landscape was snow covered; no

Table 1. Multiple regression analyses of oxygen consumption and field metabolic rate (FMR) as functions of time and body mass. $Sp\ VO_2 = ml\ O_2 \cdot kg^{-1} \cdot hr^{-1}$; $VO_2 = ml\ O_2 \cdot hr^{-1}$; $FMR\ (measured\ FMR) = LCO_2 \cdot d^{-1}$; $Sp\ FMR = LCO_2 \cdot kg^{-1} \cdot d^{-1}$. $D = Julian\ date$.

Animal Group	Regression	p Regression	R^2	N	Partial p	
					Date	Mass
Young	$VO_2 = 1322 - 6.07D + 260\ kg$	0.0001	0.62	21	0.0001	0.0001
	$Sp\ VO_2 = 838 - 2.98D + 3.76\ kg$	0.0001	0.68	21	0.0001	0.23
Yearlings	$VO_2 = 797 - 3.02D + 141\ kg$	0.0001	0.57	39	0.0001	0.0001
	$Sp\ VO_2 = 538 - 1.32D + 19.8\ kg$	0.0001	0.71	39	0.0001	0.0001
Adult females	$VO_2 = 379 - 7.84D + 103\ kg$	0.0001	0.66	25	0.0001	0.061
	$Sp\ VO_2 = 308 - 2.12D - 27\ kg$	0.0001	0.71	25	0.0001	0.081
	$FMR = 115 - 0.751D + 32.2\ kg$	0.042	0.33	19	0.015	0.096
	$Sp\ FMR = 57.5 - 0.216D + 2.36\ kg$	0.011	0.43	19	0.024	0.686
Adult males	$VO_2 = 1302 - 4.65D + 100\ kg$	0.001	0.32	38	0.004	0.011
	$Sp\ VO_2 = 550 - 1.26D + 24.9\ kg$	0.001	0.31	38	0.004	0.02
	$FMR = 78.5 + 1.59D - 0.03\ kg$	0.002	0.31	37	0.0001	0.086
	$Sp\ FMR = 0.6 + 0.515D - 0.018\ kg$	0.001	0.33	37	0.0001	0.003

foraging was possible. Because foraging on new vegetation probably reactivates the shrunken gastrointestinal tract (Hume et al., 2002), reduced VO_2 is not surprising since the gastrointestinal tract is a high energy user. We could not test whether foraging increased VO_2 because the female became coyote prey. The seasonal decline in VO_2 is associated with a seasonal decline in foraging time in nonreproductive adult females ($p = 0.12$, $R^2 = 0.77$) but not in reproductive females ($p = 0.99$, $R^2 = 0$), who allocate about the same amount of time to foraging throughout the summer. Adult males have maximal VO_2 in late June and July after mating is concluded but when intrusions into their territories by other males are maximal (Salsbury and Armitage, 1994). The decline in male intrusions as summer progresses is reflected in the decline in VO_2 (Fig. 4). The higher VO_2 of males than of females is consistent with the higher daily activity of males (Armitage et al., 1996) and is consistent with the interpretation that activity is a primary factor in the metabolic differences between males and females (Salsbury and Armitage, 1994).

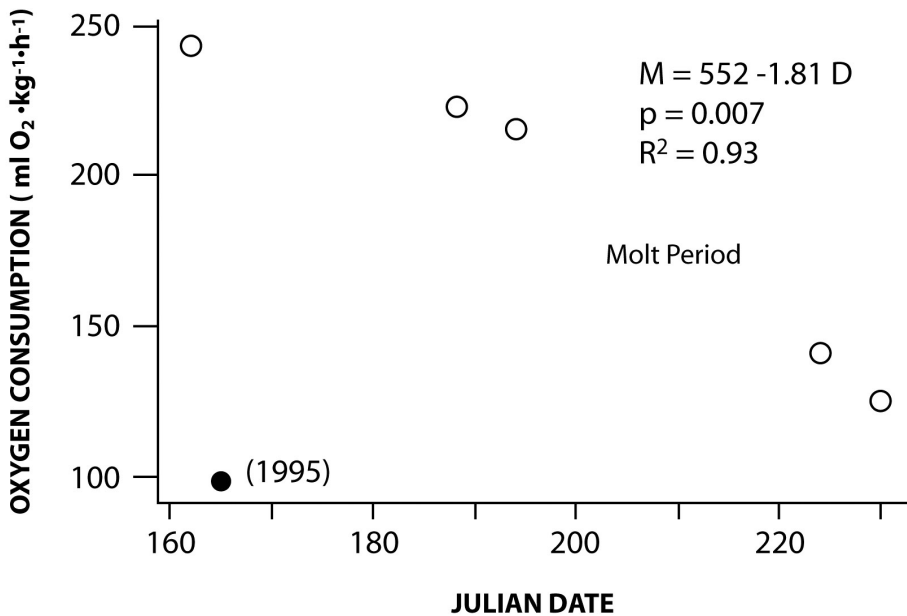


Fig. 3. The relationship between specific metabolic rate and time for an adult female. $\text{Mass (Kg)} = -1.36 + 0.0237 D$; D = Julian date.

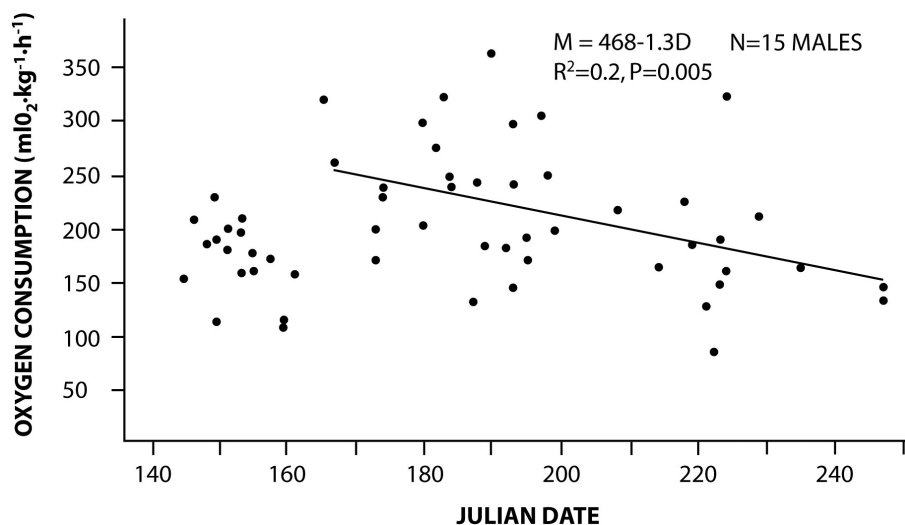


Fig. 4. Seasonal change in metabolic rate of adult males. Each point represents one measurement. Body mass varied from 1.95 to 6.3 Kg.

Effect of Drought

A late summer drought in 1991 markedly reduced survival of young and reproductive females (Armitage, 1994). We tested the prediction that marmots reduced their metabolism to conserve energy by comparing specific VO_2 of marmots in the drought year with the years before and after the drought. Because of small sample size of adult females, the two nondrought years were combined. All measurements were made after day 220. The prediction was not supported. In the drought year, mean specific VO_2 of young (322.7 ± 9.4 , $N = 19$) was significantly greater ($p = 0.004$) than VO_2 in the year before the drought (265.4 ± 11.1 , $N = 18$) and after the drought (286.4 ± 12.4 , $N = 18$). For adult females, VO_2 was higher in the drought year (197.8 ± 11.8 , $N = 15$) than in the nondrought years (170.7 ± 10.9 , $N = 17$), but $p = 0.102$. Adult females may have been less affected by the drought because nonreproductive females had mostly completed mass gain by the time of the drought (Armitage, 1994, 1996). Although water content of the plants was not measured, the plants were visibly drier; e.g., wilting and leaf necrosis. Similarly, water voles on a high cellulose diet increased metabolic rate (Woodall, 1989). Apparently foraging on drier plants increased VO_2 . Because drought affects mortality and reproduction in

several marmot species (Armitage and Blumstein, 2002), the effect of water content of food on metabolism of marmots requires more study.

Field Metabolic Rate (FMR)

Predicted FMR ($\text{L CO}_2 \cdot \text{d}^{-1}$) for adult nonreproductive females was significantly higher ($t = 3.4$, $p < 0.01$, $N = 17$) than measured FMR (100.7 vs 78.6). Neither measured FMR ($\text{L CO}_2 \cdot \text{d}^{-1}$, $p = 0.75$) nor specific FMR ($\text{L CO}_2 \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, $p = 0.09$) were significantly related to body mass. For reproductive females, predicted mean FMR (95.7) did not differ significantly ($p > 0.5$) from mean measured FMR (102.2). When lactation values were removed, mean measured FMR (62.4) was significantly lower ($t = 2.95$, $p < 0.05$) than mean predicted value (93.3). During lactation, mean measured FMR (149.0 ± 23.6) was much greater ($t = 2.7$, $0.1 > p > 0.05$, $N = 4$) than mean predicted FMR (97.4 ± 3.42). In reproductive females, specific FMR was unrelated to body mass ($p = 0.48$), but FMR was ($p = 0.053$, $R^2 = 0.65$). Thus the larger reproductive females expended more total energy but not more per body mass. FMR increased to a peak during lactation and declined thereafter (Fig. 5).

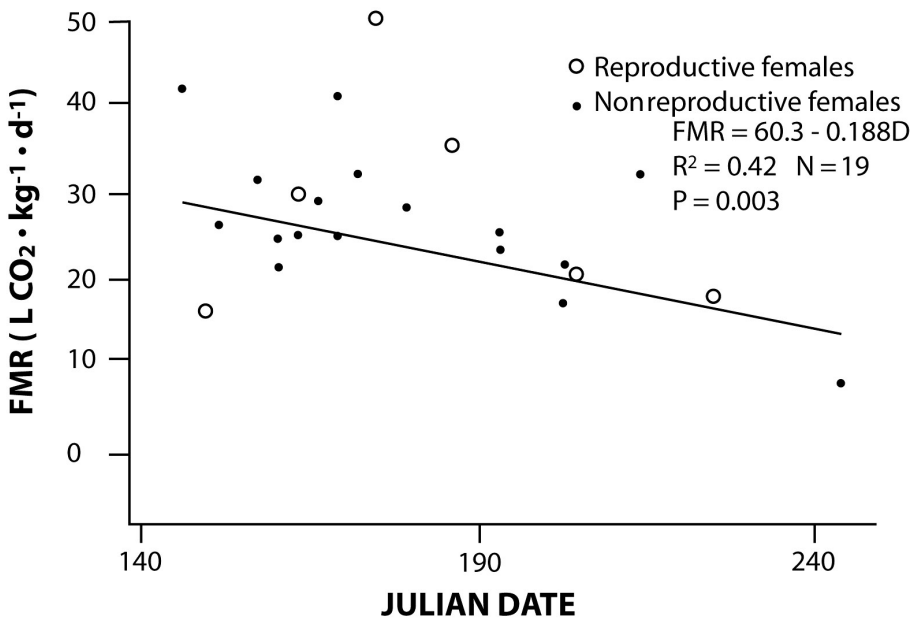


Fig. 5. Seasonal change in specific field metabolic rates (FMR) of adult females. Body mass varied from 2.21 to 3.93 Kg.

When the effects of lactation are excluded from the analysis, there is a significant seasonal decline in FMR and specific FMR (Fig. 5, Table 1). Body mass contributed little to the seasonal pattern of FMR. The seasonal decline in both measures of FMR is consistent with the seasonal decline in VO_2 (Fig. 2) and may reflect the expression of the circannual metabolic rhythm (Ward and Armitage, 1981; Armitage and Woods, 2003).

Mean measured FMR (86.4) of adult males did not differ ($t = 1.2$, $p > 0.2$, $N = 37$) from mean predicted FMR (96.4). The 10 highest measured FMR occurred when each male was in conflict with other adult or yearling males; four were new invaders at their sites, and two of these dispersed. Mean measured FMR of conflict males was 65.7% larger than predicted FMR. If the remaining measurements are considered separately, mean measured FMR decreases to 63.4 and is significantly lower ($t = 5.3$, $p < 0.001$, $N = 27$) than mean predicted FMR (95.9). In multiple regression analysis, both measured FMR and specific FMR were related to mass and both increased seasonally (Table 1). Measurements of FMR of conflict males occurred on average 18 days later than those of the other males ($t = 2.2$, $0.05 > p > 0.02$). For each group of males, FMR was regressed against Julian date and the equations were used to predict FMR for day 175, when FMR was highest for the nonconflict males and at the middle of the time period for conflict males. The predicted FMR of 149.0 for conflict males was 97.6% greater than the FMR of 75.4 for nonconflict males. Thus neither body mass nor season accounts for the differences in FMR of the two groups of males. The regression for conflict males explained only 11% of the variation in FMR; individual conflict males may have high values at any time during the summer depending on the presence of intruder males.

Specific FMR did not differ between adult males and females when all values were included ($t = 1.4$, $p > 0.1$) or when reproductive females and conflict males were removed from the analysis ($t = 0.6$, $p > 0.5$). Neither measured FMR (148.6 vs 149.0) nor specific FMR (47.6 vs 47.3) differed between conflict males and lactating females. These results suggest that there is no difference in the reproductive cost of females and males. The nature of the cost differs between the sexes; for females it is lactation; for males, territorial conflict. However, the total annual costs may be greater for males. Reproductive females have a high FMR for about 30 days (Fig. 5) whereas conflict males may have high FMR for up to 60 days. Therefore, specific FMR was compared between nonreproductive females and conflict males over the same time period. Values for females were chosen from a date that was identical or close to the date for

which a male value was available. Mean specific FMR of 10 conflict males (47.6 ± 5.8) was significantly ($t = 3.6$, $p < 0.01$) greater than that of 10 females (25.0 ± 2.6). One major reason for the difference is that specific FMR of the females declined ($p = 0.098$, $R^2 = 0.32$) over the 60-day period whereas specific FMR of males did not change ($p = 0.9$, $R^2 = 0.001$). The higher specific FMR of males is consistent with the higher metabolic rate of males than females, the greater amount of time spent above-ground by adult males (Armitage et al., 1996), and may explain the higher rates of mortality and shorter life span of adult males (Schwartz et al., 1998). Similarly, mortality is higher in male than in female *S. beldingi* and *S. richardsonii* in part because of injuries in fights with other males over access to females (Yensen and Sherman, 2003).

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Metabolic Rate Reduction During Hibernation and Daily Torpor

FRITZ GEISER

Centre for Behavioural and Physiological Ecology, Zoology, University of New England,
Armidale, NSW 2351, Australia

Abstract. Mechanisms causing the substantial reduction of metabolic rates (MR) during torpor (TMR) in mammals and birds remain controversial. It has been suggested that body temperature (T_b), metabolic inhibition, the small differential between T_b and ambient temperature (T_a), or low thermal conductance are responsible for the low TMR. Available data suggest that MR reduction depends on patterns of torpor, state of torpor, and body mass. Daily heterotherms (species displaying daily torpor exclusively) appear to rely to a large extent on the fall of T_b for MR reduction, perhaps with the exception of very small species and at high T_b during torpor, where some metabolic inhibition may be used. In contrast, hibernators (species capable of prolonged torpor bouts) rely extensively on metabolic inhibition, in addition to T_b effects, to reduce MR to a fraction of that observed in daily heterotherms. In small hibernators metabolic inhibition and the large fall of T_b are employed to maximise energy conservation, whereas in large hibernators metabolic inhibition appears to be employed to facilitate MR and T_b reduction at torpor onset. Over the T_a range where torpid heterotherms are thermo-conforming, the $T_b - T_a$ differential is more or less constant despite a decline of TMR with T_a . However, in thermo-regulating torpid individuals, the $T_b - T_a$ differential is maintained by a proportional increase of TMR as during normothermia, albeit at a lower T_b . Thermal conductance in most torpid thermo-regulating individuals is similar to that in normothermic individuals despite the substantially lower TMR in the former; however, conductance is low when deeply torpid animals are thermo-conforming, likely because of peripheral

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

vasoconstriction. Consequently most of the apparently contradictory hypotheses that have been proposed to explain metabolic rate reduction appear to be correct. However, not all hypotheses match the measured data of all species in all stages of torpor.

Introduction

Most researchers working on daily torpor and hibernation agree that the reduction of MR during torpor is substantial and is pivotal for survival in many species. Nevertheless, the mechanisms of how the MR is reduced remain controversial. Several and at first glance mutually exclusive hypotheses attempting to explain the MR reduction during torpor have been proposed. These hypotheses suggest that MR is reduced: (1) via temperature effects (Hammel et al., 1968; Snapp and Heller, 1981), (2) by physiological inhibition in addition to temperature effects (Malan, 1986; Geiser, 1988; Storey and Storey, 1990), (3) by the small $T_b - T_a$ differential (Heldmaier and Ruf, 1992), or (4) the low apparent thermal conductance (C) in torpid individuals (Snyder and Nestler, 1990). These hypotheses are examined here in relation to patterns of torpor, state of torpor, and body mass, because these attributes appear important in determining MR and T_b during torpor.

Data Selection and Analysis

Data on MR, T_b , and body mass of heterothermic mammals and birds were collected from the literature. Basal MR (BMR) was used as a reference point for the TMR of thermo-conforming torpid individuals because in both physiological states metabolism is used for maintenance only, without extra energy expenditure for thermoregulation (Bucher and Chappell, 1997; Wang and Lee, 2000). TMR data were statistically analysed in different T_b bins of 0.0–9.9° C, 10.0–14.9° C, 15.0–24.9° C, and 24.0–32.9° C and the TMR and the Q_{10} (the change in rate caused by 10° C change in temperature) was calculated between BMR (MR_1) and TMR (MR_2) at corresponding T_b s ($Q_{10} = MR_1/MR_2^{10/(T_{b1}-T_{b2})}$) and analysed as a function of body mass. Data for torpid individuals were collected at T_a below and above the T_b set point (T_{set}) during torpor to examine relations between MR and T_b , MR and C, and MR and the $T_b - T_a$ differential in daily heterotherms and hibernators (further details and data in Geiser, 2004).

Torpor Entry

At torpor entry the T_{set} falls faster than T_b facilitated by thermal inertia (Heller et al., 1977). Since most species enter torpor at low T_a , well below the thermoneutral zone (TNZ), the fall of T_{set} should result in a fall from resting MR (RMR) to \sim BMR, because heat production for normothermic thermoregulation will cease (Withers 1992). In sugar gliders, *Petaurus breviceps*, a $\sim 4^\circ\text{C}$ drop from a nocturnal T_b to a diurnal resting normothermic T_b results in a precipitous drop from RMR to \sim BMR, which superficially appears to be a torpor entry (Fig. 1), supporting the theoretical prediction.

The initial reduction of MR at torpor onset at low T_a in most species will follow a similar pattern (Song et al., 1996). However, because the T_{set} is reduced by $> 4^\circ\text{C}$, the substantial fall in T_b that must follow the reduction from RMR to \sim BMR at torpor onset of most heterothermic species is one of the reasons why MR can fall to well below the BMR.

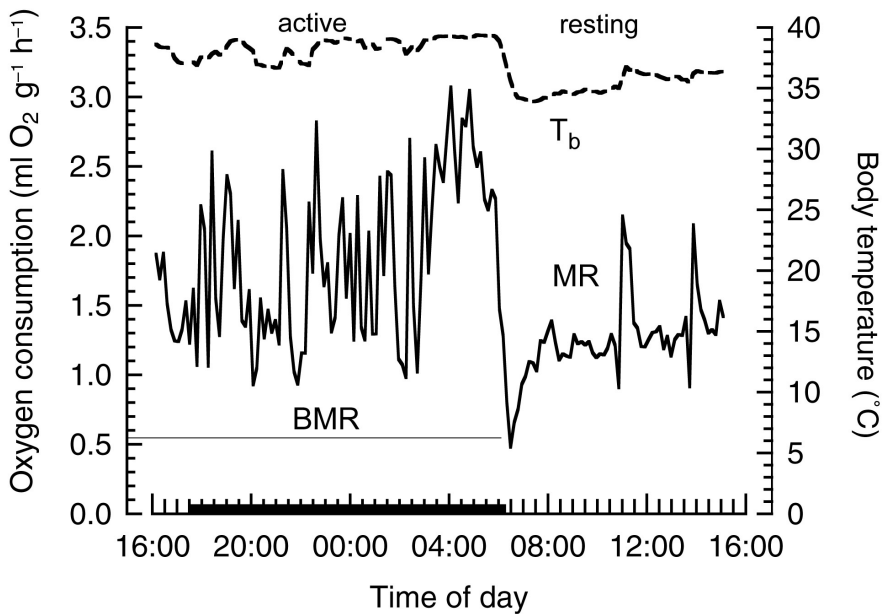


Fig. 1. Oxygen consumption of a sugar glider (*Petaurus breviceps*, 120 g) exposed to T_a 10°C during the activity phase at night (dark bar) and the rest phase at daytime. Note the transient, precipitous drop of oxygen consumption to near BMR during the cooling phase from activity phase to rest phase body temperatures near lights on, followed by a return to RMR after the cooling phase (Holloway, 1998).

Obviously, the scope for the reduction of RMR depends on size. Small mammals and birds (10 g) have a high RMR at low T_a and a fall of T_{set} will result in a large reduction of MR from RMR to BMR (Fig. 2). The substantial reduction of MR together with the large relative surface area of small heterotherms will result in high cooling rate, and the fast fall of T_b will in turn affect MR. In contrast, in medium (250 g) or large heterotherms (5,000 g) the TNZ extends to a lower T_a , RMR at low T_a increases only little above BMR (Fig. 2), and their small relative surface area will result in a slow cooling rate. Very large species, such as bears, are under thermoneutral conditions even at T_a near 0° C (Scholander et al., 1950) and a fall of T_{set} under thermoneutral conditions should have no effect on MR.

Thus, physiological mechanisms employed for MR reduction during torpor entry must differ between small and large heterotherms. Small species are able to reduce MR with a fall of T_b . In contrast, large species cannot rely on T_b , at least

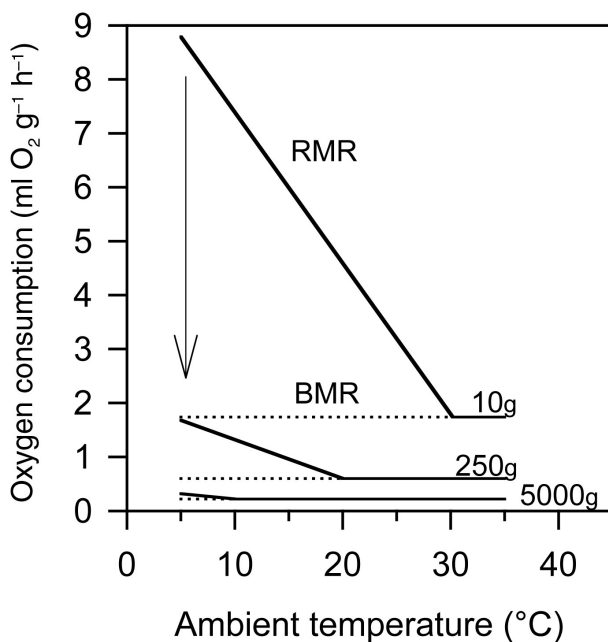


Fig. 2. Resting metabolic rate within the TNZ (BMR) and below the TNZ (RMR) in endotherms of different body mass. Note the wide TNZ and the small increase from BMR to RMR in the large in comparison to the small species. This difference will affect MR reduction at torpor onset (arrow).

not in the initial phase of torpor entry, and metabolic inhibition for MR reduction appears unavoidable.

Allometry of Steady-state BMR and TMR of Thermo-conforming Torpid Animals

Different approaches to MR reduction as a function of size are not only observed during torpor entry but also are reflected in the steady-state TMR and the Q_{10} between BMR and TMR. Moreover, TMR differs between daily heterotherms and hibernators.

In daily heterotherms at all T_b ranges examined, the regression lines for TMR as a function of body mass declined in parallel with T_b (Fig. 3A). However, the elevation (y-intercept) differed between BMR and TMR at T_b 25–33° C, and also between TMR at T_b 25–33° C and T_b 15–25° C (ANCOVA: $p < 0.001$).

Hibernators also reduce MR as a function of mass from BMR to TMR in parallel from normothermic T_b down to T_b 15–25° C (Fig. 3B). At high T_b s the slopes of the regression for BMR and TMR were indistinguishable, but at $T_b < 10°$ C the slope for the regression of TMR vs mass became significantly smaller ($p < 0.024$; ANCOVA). Above T_b 15° C, the slopes for mass-specific TMR versus body mass ranged from -0.214 to -0.304 ; below T_b 10° C, the slope was \sim half (-0.128) because in this T_b range the reduction of TMR relative to BMR in the small species is more pronounced than in the large species.

When the TMR in the T_b bins of daily heterotherms (Fig. 3A) and hibernators (Fig. 3B) were compared, all differed significantly in elevation (ANCOVA $p < 0.0001$) at T_b 25–33° C, T_b 15–25° C, and T_b 10–15° C. These differences were not due to differences in T_b , because mean T_b s were indistinguishable.

The Q_{10} Between BMR and TMR in Thermo-conforming Torpid Animals

The relationships between TMR and body mass are reflected in the Q_{10} (Fig. 4). As the TMR in daily heterotherms was relatively high, the Q_{10} values between BMR and TMR at all T_b during torpor were significantly smaller than in hibernators. In daily heterotherms the Q_{10} values were similar among the different T_b ranges (T_b 10–15° C, $Q_{10} = 2.0 \pm 0.2$; T_b 15–25° C, $Q_{10} = 2.4 \pm 0.7$; T_b 25–33° C, $Q_{10} = 2.3 \pm 0.7$) and the overall mean Q_{10} was 2.3 ± 0.6 ($n = 49$ species) close to those typical for biochemical reactions. The Q_{10} values for hibernators ranged from 2 to 27 (2 to 9.7 without the bear [*Ursus americanus*], which appears an overestimate). In hibernators, Q_{10} values increased with T_b (T_b 10–15° C, Q_{10}

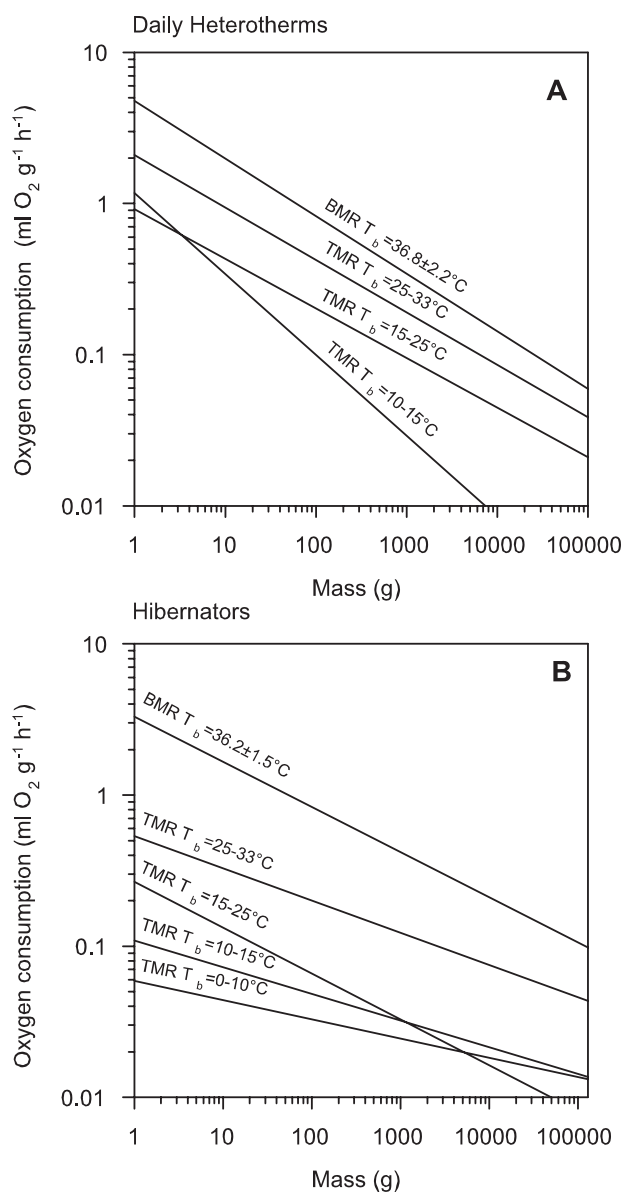


Fig. 3. MR as a function of body mass for daily heterotherms (A) and hibernators (B) at different T_b . Only regressions lines are shown for clarity. All regressions are significant with the exception of that for hibernators at T_b 10-15°C ($r^2 = 0.24$, $p = 0.09$). Note the big differences in elevation (y-intercept) between A and B for regression lines fitted to torpid individuals at $T_b < 33^\circ\text{C}$.

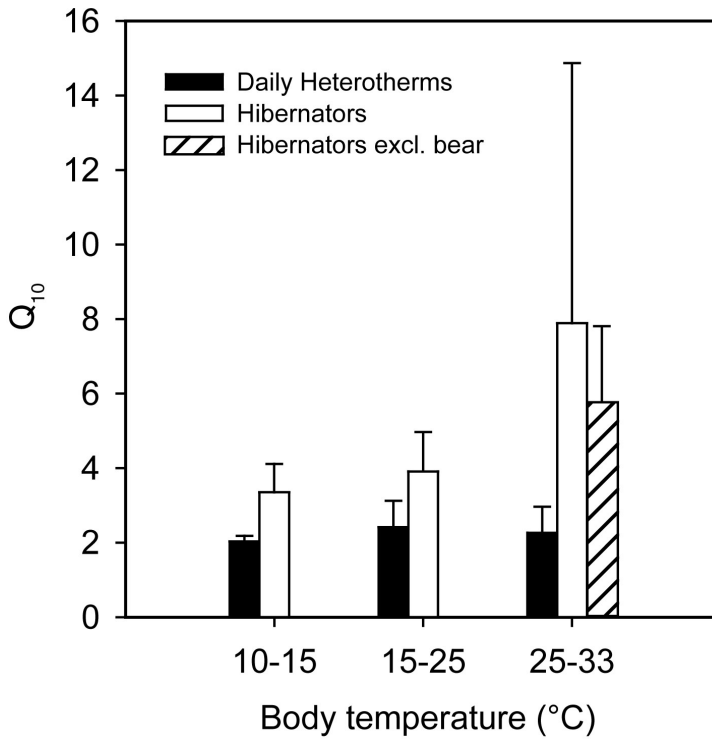


Fig. 4. Q_{10} values for daily heterotherms and hibernators between BMR and TMR measured at different T_b . Q_{10} values for daily heterotherms were not affected by T_b and were close to those typical for biochemical reactions (average $Q_{10} = 2.3 \pm 0.6$). In contrast, Q_{10} values in hibernators increased with T_b from $Q_{10} = 3.4 \pm 0.8$ at low T_b to $Q_{10} = 7.9 \pm 7.0$ ($Q_{10} = 5.8 \pm 2.0$ excl. bear) and the average Q_{10} was 3.4 ± 0.9 (excl. bear).

$= 3.4 \pm 0.8$; T_b 15–25° C, $Q_{10} = 3.9 \pm 1.1$; T_b 25–33° C, $Q_{10} = 7.9 \pm 7.0$) and the overall Q_{10} was 3.9 ± 3.7 ($n = 43$ species; 3.4 ± 0.9 without the bear), well above those typical for biochemical reactions. The Q_{10} values differed significantly between daily heterotherms and hibernators in the three T_b ranges (Fig. 4) as well as the overall Q_{10} for the 92 species examined ($p < 0.001$).

At $T_b < 10^\circ$ C, the Q_{10} values of hibernators were negatively related to body mass ($r^2 = 0.28$), reflecting a greater reduction of steady-state TMR below BMR in the small species in comparison to the large species at low T_b (Fig. 3B). At T_b 10–15° C and T_b 15–25° C, Q_{10} values between BMR and TMR of hibernators were not affected by body mass.

TMR and the $T_b - T_a$ Differential

Thermo-regulating torpid individuals at T_a below the T_{set} for T_b maintain TMR according to the $T_b - T_a$ differential, albeit at a lower T_b than during normothermia (Hainsworth and Wolf, 1970). Obviously, regulation of T_b even during torpor will result in a proportional heat loss as during normothermia, which must be compensated for by an increase in heat production.

In contrast to thermo-regulating torpid individuals, the $T_b - T_a$ differential in thermo-conforming individuals is often constant (~ 1 to 3°C) or changes little with T_a , although TMR shows a significant decline with T_a and consequently T_b (Hock, 1951; Henshaw 1968; Song et al., 1997; Buck and Barnes, 2000; Wang and Lee 2000). These observations show that above the T_{set} the $T_b - T_a$ differential does not determine steady-state TMR in thermo-conforming torpid heterotherms.

Apparent Thermal Conductance (C) and TMR

Snyder and Nestler (1990) proposed that a low C rather than a change of T_b allows endotherms to markedly reduce MR without abandoning regulation of T_b during torpor. This argument has several problems: (1) C during torpor in most species is lower only when torpid animals are thermo-conforming and apparently do not regulate T_b , (2) the reduction of C from RMR to TMR is small in comparison to the large difference in metabolism, (3) exposure to 21% oxygen in helium, which is a more conductive atmosphere than air and will increase C, does not result in an increase in TMR (Geiser et al., 1996), (4) C of most thermo-regulating animals is the same during torpor and normothermia (Song et al., 1997; Geiser, 2004), but the TMR even in thermoregulating individuals is only a fraction of that during normothermia. Thus, a low C cannot be the reason for the low MR (Nicol et al., 1992), but appears to be a consequence of the low TMR and peripheral vasoconstriction.

Are Q_{10} Calculations Meaningful in Endotherms?

As for all other physiological measurements, common sense must be applied to the calculation of Q_{10} (Wang and Lee, 2000). This is especially important in heterothermic endotherms, which exhibit pronounced changes in their physiological state. If meaningful calculations for Q_{10} are to be made, changes of MR with T_b that are based on equivalent performance at different T_b s appear most appropriate (Wang and Lee, 2000). Thus, in the present comparison, Q_{10} values were calculated between BMR and TMR in thermo-conforming individuals

because both states do not include a thermoregulatory energetic component and reflect maintenance metabolism only at different T_b . Calculations of Q_{10} values between TMR at different T_b during torpor in thermo-conforming individuals also are appropriate. In contrast, comparisons of thermo-regulating individuals (apples plus oranges) with thermo-conforming individuals (apples) are not likely to provide a meaningful Q_{10} values, because a change of state rather than the effects of temperature on rates are examined. Similarly, calculations of Q_{10} during torpor entry can be meaningless if they fail to consider that the initial decline of MR is often not related to a reduction of T_b , but a reduction of T_{set} .

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How to Enter Torpor: Thermodynamic and Physiological Mechanisms of Metabolic Depression

GERHARD HELDMAIER¹ AND RALF ELVERT²

¹ Department of Biology, Philipps University Marburg, Marburg, Germany

² German Mouse Clinic, GSF, D-85764 Neuherberg, Germany

Abstract. Dormice (*Glis glis*) enter torpor at ambient temperatures from 2 through 30° C. At temperatures below thermoneutrality they display hibernation or daily torpor, at thermoneutral temperatures (>22.4° C) they showed daily torpor. We recorded metabolic rate and body temperature during spontaneous entries into torpor and analysed the contribution of active metabolic inhibition and temperature effects during metabolic depression. Entrance into torpor is initiated by a metabolic inhibition below the level of basal metabolic rate. Final depression of metabolic rate is accomplished by a synergistic action of temperature effects and metabolic inhibition. In hibernation (four species) the contribution of metabolic inhibition is about 70% and the role of temperature effects about 30%, whereas in daily torpor (two species) both mechanisms contribute by about 50% to metabolic depression.

Introduction

The physiological mechanism of metabolic depression in hibernation is still unknown, and two alternative paradigms have been developed. The “classical view” suggests that low body temperature in hibernation decreases biochemical reaction rates and thus allows hibernators to survive with a fraction of their euthermic energy requirements. Evidence for this response is based on the observation that the setpoint (T_{set}) for body temperature (T_b) is downregulated during entrance into hibernation (Heller et al., 1977). This causes a decrease in T_b , which consequently lowers metabolic rate, i.e., hypothermia is the cause of depressed

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

metabolism in hibernation. The relations between T_b and metabolic rate in hibernation reveal a Q_{10} of 2–3 in several species, supporting the view of enzymatic thermodynamic suppression of metabolic rate in torpor (Geiser, 1988; Song et al., 1997; Zimmer and Milsom, 2001). The “alternative view” suggests that hibernators and torpidators actively inhibit their metabolic rate during entrance into hibernation. This reduces endogenous heat production and T_b decreases. Thus low T_b is the consequence but not the cause of metabolic depression (Heldmaier and Ruf, 1992). Evidence for this alternative view is derived from the observation that reductions of metabolic rate anticipate the development of hypothermia during entrance into torpid states. Q_{10} s calculated for this depression may reach values > 20 . Furthermore, the Q_{10} for metabolic depression is inversely correlated with body size, calculating Q_{10} ’s of up to 6 in small mammals, which is far above thermodynamic effects on respiration, suggesting a physiological inhibition of metabolic rate in small mammals (Geiser, 1988).

The controversy partly originates from the fact that low metabolic rate in hibernation is always associated with a lowered T_b . Therefore, the effect of metabolic inhibition cannot easily be separated from thermodynamic influences on metabolic rate, and it is difficult to estimate the contribution of either metabolic or thermodynamic responses. Furthermore, the occurrence of a Q_{10} of 2–3, which is the common range for temperature effects on biochemical reactions, does not prove thermodynamic effects of T_b on metabolic rate, because this Q_{10} may also be generated by thermoregulatory responses to changes in T_{set} at constant T_a or to changes in thermal conductance (Snyder and Nestler, 1990; Heldmaier and Ruf, 1992). The limitations of an experimental analysis of temperature-metabolism relations in torpor have been summarized by Wang and Lee (2000), concluding that the “use of Q_{10} is fraught with pitfalls both in its application and its interpretation.”

The answer to the question of whether hibernators actively inhibit their metabolic rate or if they only depend on temperature effects is of basic significance for our understanding of the physiology of torpid states. In the latter case we can assume that hibernators and torpidators only specialized their thermoregulatory system in order to allow and tolerate controlled hypothermia, causing a decrease of metabolic rate. Alternatively, hibernators and torpidators should be considered as endotherms that can actively control their metabolic rate in euthermia, in torpor, and during transition between the two states. They can deliberately switch between their tachymetabolic state in euthermia and their bradymetabolic state in torpor. The control of basal metabolic rate (BMR) in endotherms,

both on the cellular as well as on the organismic level, is one of the large unknowns in animal physiology. Hibernation is a unique behaviour that could help us to unravel the mystery of metabolic rate control in endotherms.

Methods

We measured metabolic rate (torpid metabolic rate, TMR) during hibernation and daily torpor in common dormice (*Glis glis*) over a wide range of T_a and T_b (0–30° C). On the basis of this large data set we tried to identify ranges of controlled regulation of metabolic rate and to separate this from temperature effects on metabolic rate. Dormice were kept in small aviaries (0.8 x 0.5 x 0.4 m) with a sleeping box attached. They could enter the sleeping box through a tube (diameter 4 cm) which was equipped with a revolving door. The sleeping box served as chamber for measurement of metabolic rate and was equipped with an infrared camera for visual observations of dormice. A receiver for telemetry of T_b was placed underneath the sleeping box. Dormice rapidly learned to use this setup and spontaneously used the sleeping box for hibernation or daily torpor. For measurement of body temperature a temperature transmitter was implanted into the abdominal cavity (Data Sciences International, Model TA10ETA-F20, weight 3.9 g). Metabolic rate and T_b were recorded continuously in 1 minute intervals, which allowed uninterrupted observations of voluntary entries into torpid states and long-term maintenance of torpid states at different T_a (Wilz and Heldmaier, 2000; Elvert and Heldmaier, 2000). Torpor bouts lasting longer than 48 hours were defined as hibernation, whereas daily torpor bouts lasted about 8 through 18 hours.

Temperature Range for Hibernation and Daily Torpor

Euthermic dormice increased their metabolic rate in the cold approximately proportionally with the increasing cold load ($MR = 1.944 - 0.058 * T_a$). The intercept of this regression with basal metabolic rate (BMR) was at T_{lc} 22.2° C, indicating a thermoneutral zone above 22.2° C T_a . The upper end of the thermoneutral zone was not determined, but exposures to 29° C caused no profound rise in metabolic rate of euthermic dormice, although they preferred a stretched posture, indicating that this temperature was close to the upper end of their thermoneutral zone. Between 2 and 20 °C T_a , dormice entered hibernation (Fig. 1). Below 2° C they did not enter hibernation spontaneously (except in one single case), but when they were already hibernating they tolerated a further slight lowering of temperature close to 0° C. If we compare the zones

of euthermic thermoregulation with the occurrence of torpid states, it can be concluded that hibernation only occurred at moderate cold exposure, i.e., at temperatures below the thermoneutral zone, whereas daily torpor occurred over a broader range of T_a from 5 through 29° C.

Torpor at Thermoneutrality

Most remarkable is the circumstance that dormice became torpid at thermoneutrality, at T_a between 23 and 29° C. Daily torpor at thermoneutrality has also been observed in Djungarian hamsters (*Phodopus sungorus*) and in the marsupial

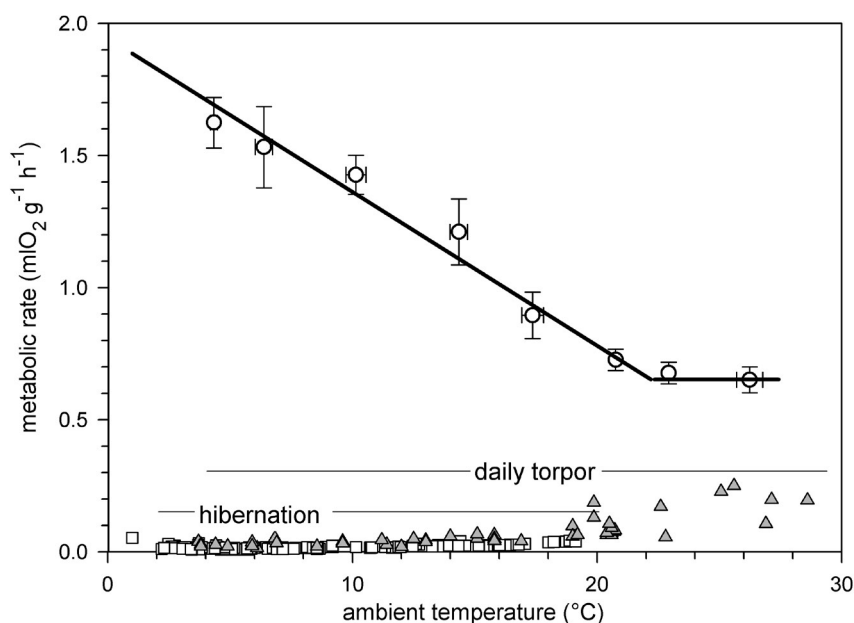


Fig. 1. Metabolic rate and the temperature range of hibernation or daily torpor in dormice. Oxygen consumption of dormice was measured as described before (Wilz and Heldmaier, 2000). Dormice were kept in a climate chamber and metabolic rate was measured continuously for several months, while T_a was varied between 0° C and 30° C. Typically, T_a was kept constant for about a week. Values for hibernation (squares, 148 hibernation bouts in seven dormice) or daily torpor (triangles, 51 daily torpor bouts in seven dormice) were only entered at temperatures at which they entered torpid states spontaneously. Circles are mean values \pm SD of metabolic rate in euthermia, T_{lc} lower critical temperature. Part of the data were published previously (Wilz and Heldmaier, 2000) and have been extended by further studies.

Cercartetus nanus (Ruf and Heldmaier, 1992; Song et al., 1997). Both are small mammals with a body mass of about 30 g. The common dormouse is much larger, with a mean body mass of 138 g, and at 29° C they already showed signs of increasing heat load. At thermoneutrality all heat produced in the body originates from obligatory heat production by BMR, and T_b is regulated by physical means of heat loss (convection, conduction, radiation, evaporation).

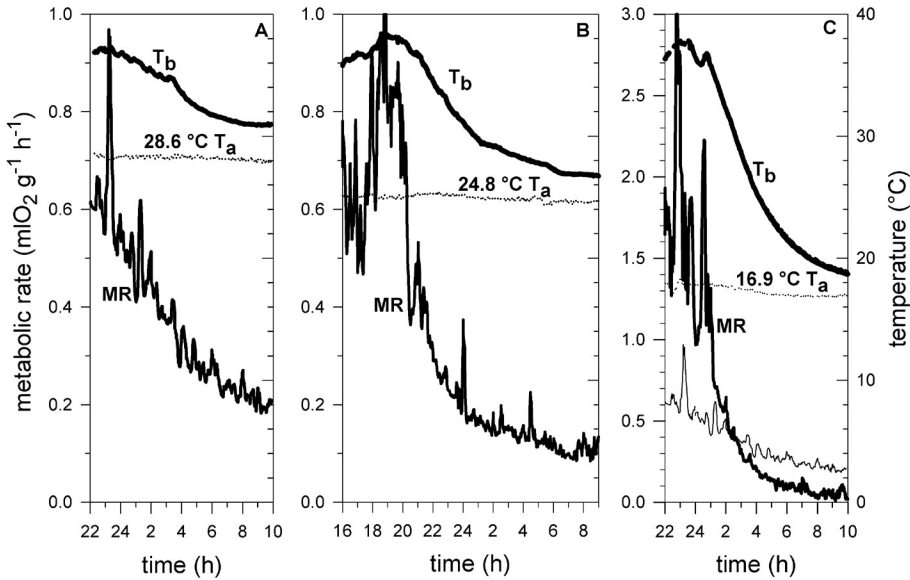


Fig. 2. Entrance into daily torpor in a common dormouse (*Glis glis*) at thermoneutrality (28.6° C T_a [A], 24.8° C T_a [B]) and during moderate cold exposure (16.9° C T_a [C]). Metabolic rate during moderate cold exposure is much higher than at thermoneutrality, therefore the scaling of the y-axis in (C) is larger than in (A) and (B). For better comparison the metabolic rate from (A) was repeated as a thin line in (C). Metabolic rate was measured in 1 minute intervals in the nesting box (2 l) at relatively high flow rates (35–60 l/h) to obtain a short lag time and high resolution. In all three examples metabolic rate is presented as 5 min moving average. T_a was measured inside the nesting box. All three entries were observed in the same individual (R13G), during three different sessions (C June 24, B August 5, A August 12). Dormice have a highly variable body weight due to rapid fat accumulation. Therefore the body weight varied during the sequence of these sessions and was 138 g (A), 111 g (B), and 122 g (C). The Q_{10} calculated for metabolic depression from the resting level to the final hour in torpor was 6.09 (A), 6.24 (B), and 6.70 (C).

Thermoregulatory heat production is absent under these conditions and cannot be suspended to initiate the development of hypothermia. Therefore, at thermoneutrality torpor can only be initiated through an active inhibition of heat production below the level of BMR.

An example of entrance into torpor at 28.6° C is presented in Fig. 2A. BMR of this dormouse was 0.601 mlO₂ g⁻¹ h⁻¹, and the beginning of entrance was marked by a peak in metabolic rate, followed by a gradual decline of metabolic rate below the level of BMR. This was paralleled by slight reduction of T_b from 36.5 to 34.3° C within about four hours. The decline of metabolic rate and T_b continued for another 6 h, and at the end of the torpor bout TMR was about one third of BMR (0.196 mlO₂ g⁻¹ h⁻¹) and T_b had reached 30.3° C. A further example in Fig. 2 shows entrance into torpor at the lower end of the thermoneutral zone (24.8° C; Fig. 2B), which caused a greater depression of metabolic rate (0.107 mlO₂ g⁻¹ h⁻¹) and T_b (26.9° C). At moderate cold (16.9° C; Fig. 2C) the dormouse developed even lower values of metabolic rate (0.0416 mlO₂ g⁻¹ h⁻¹) and T_b (17.5° C). All three torpor bouts were terminated by an arousal at the end of the records shown in Fig. 2.

Thermal conductance was calculated from metabolic rate and the T_b-T_a gradient for euthermic dormice prior to entrance into torpor and at the end of the torpor bout. Corresponding values (mlO₂ g⁻¹ h⁻¹ ° C⁻¹) are 0.076 versus 0.080 at 28.6° C T_a (A), 0.065 and 0.051 at 24.8° C T_a (B), and 0.080 versus 0.074 at 16.9° C T_a (C), concluding that thermal conductance was not profoundly altered in the torpid state. However, estimates of thermal conductance from metabolic rate and the T_b-T_a gradient have to be treated cautiously, because it requires a steady state of thermoregulation, and transients may cause errors. Temperature gradients in torpor are very small, often < 1° C, and small inaccuracies in T_b measurement may mimic large changes in conductance. The present finding that thermal conductance remained constant, or decreased slightly in the torpid state, is in accordance with previous measurements of conductance by direct calorimetry in hibernating *Spermophilus lateralis* (Snapp and Heller, 1981) and during daily torpor in *Phodopus sungorus* (Heldmaier and Ruf, 1992).

During entrance into torpor at thermoneutrality, T_b and metabolic rate decreased in parallel, and one could be tempted to assume that metabolic depression occurred due to thermodynamic effects of hypothermia (Fig. 2A). However, a Q₁₀ of 8.11 and 6.09 was calculated for the initial four hours and the total entrance, respectively, which is much higher than a Q₁₀ of 2–3 typically found for respiration in biological systems. Similar temperature-metabolism

relations were also found in other entries into torpor (Fig. 2), concluding that metabolic rate is lowered more than expected from thermodynamic relations, suggesting a temperature-independent inhibition of metabolic rate.

Metabolic Regulation in Deep Hibernation

In a cold environment, torpor is characterized by a greater reduction of metabolic rate and a more pronounced hypothermia (Fig. 2C). To further examine the relation between T_b and metabolic rate in torpid states, we compared hibernation and daily torpor in the entire temperature range where this behaviour occurred spontaneously (Fig. 3). In hibernating dormice means of T_b and metabolic rate were calculated for periods of 08:00 through 18:00 hours, typically during the hours preceding a spontaneous arousal. The long periods of time were chosen to obtain steady-state values and to eliminate short-term changes due to their

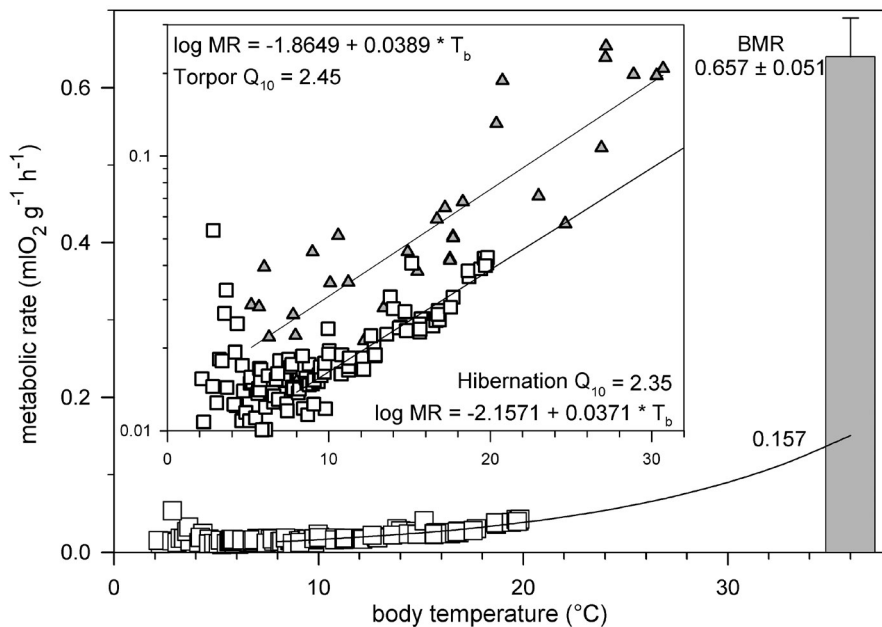


Fig. 3. Metabolic rate of dormice during hibernation (squares) and daily torpor (triangles) as related to T_b . Data are plotted with a linear scale for metabolic rate for hibernation, and in the insert with a log scale for hibernation and daily torpor. A smaller number of data are presented as used in Fig. 1 because of occasional failures of T_b telemetry (hibernation $N = 7$, $n = 117$ ($n = 63$ for regression), daily torpor $N = 7$, $n = 42$).

episodic breathing pattern. In daily torpor the mean values for T_b and metabolic rate were calculated for the last hour of the torpor bout just preceding the arousal.

Lowest values of metabolic rate are observed in hibernation, at T_b s between 2 and 12° C ($0.0151 \pm 0.0030 \text{ mlO}_2 \text{ g}^{-1} \text{ h}^{-1}$). The absence of any relation between T_b and metabolic rate in this temperature range indicates that metabolic rate is controlled at a constant level, and thermodynamic effects of T_b are compensated in deep hibernation. This finding is in agreement with observations in other species. In the echidna and ground squirrels, minimum TMR is maintained at T_b from 4–14° C, in arctic squirrels from 4–16° C, and in alpine marmots from 6–18° C (Nicol et al., 1992; Heldmaier et al., 1993; Buck and Barnes, 2000; Ortmann and Heldmaier, 2000). This can only be achieved by a compensation of temperature effects to maintain constant TMR in deep hibernation, independent from moderate changes in T_b , which may be imposed by thermal changes in the hibernaculum. This allows hibernators to endure extended periods of hibernation with minimum energy cost, even during fall and spring when temperatures in their hibernacula are above the seasonal minimum (Arnold et al., 1991).

At T_b below 4° C, some hibernating dormice did not maintain minimum TMR but increased their metabolic rate for thermoregulatory heat production. This is well documented in many species of hibernators. In fact hibernators maintain peripheral and central thermosensitivity, and they are capable of integrated thermoregulatory responses in deep hibernation (Lyman and O'Brien, 1974; Heller and Colliver, 1974; Heller et al., 1977). If T_b decreases below threshold levels, hibernators raise their metabolic rate to maintain a constant T_b in deep hibernation or to prevent a decrease of T_b below preferred levels. Thermoregulatory responses are similar to those in euthermia, except that in hibernation they regulate for a lowered T_{set} (Heller and Colliver, 1974; Ortmann and Heldmaier, 2000). This suggests that in hibernation the endothermic capability is maintained, except that the level of metabolic rate has changed from the tachymetabolism in euthermia to bradymetabolism in hibernation. If we continue this thought further, it concludes that hibernators should also be considered as endotherms while in deep hibernation.

Temperature Effects on Metabolic Rate in Hibernation

At T_b above 12° C ($T_a > 11° \text{ C}$) hibernating dormice had a higher TMR. It increased with T_b in a rather steady manner, and there was almost no individual variation in TMR as compared to hibernation at T_b below 10° C (Fig. 3, insert). The relation between metabolic rate in hibernation and T_b above 12° C revealed

a Q_{10} of 2.35. This is within the range of 2–3 biochemical temperature effects. Similar relations between T_b and TMR were also found in other hibernators above 12° C (Table 1), suggesting a thermodynamic control of TMR in deep hibernation under these intermediate thermal conditions. An alternative explanation would be that TMR is elevated for thermoregulatory control of an increasing $T_b - T_a$ gradient. The increase of the $T_b - T_a$ gradient at these intermediate temperatures in dormice was at the borderline of statistical significance (0.47 ± 0.26 at 9.7° C T_a ; 0.73 ± 0.12 ° C at 18.5° C T_a ; $p = 0.035$ ($n = 25$); correlation of $T_b - T_a$ over T_a n.s.), indicating that the changes in TMR are more likely to be explained by thermodynamic temperature effects.

Therefore we can identify three different states of metabolic rate control in deep hibernation: (1) a range of T_b where constant minimum TMR is

Table 1: Contribution of temperature effects to metabolic depression in torpor. BMR basal metabolic rate in euthermia. TMR_{min} minimum metabolic rate in torpor. TMR at body temperatures >8° C ($TMR_{>8}$) was extrapolated to euthermic body temperature of 36.5° C (TMR_{expo}).

	body mass (g)	BMR (mlO ₂ g ⁻¹ h ⁻¹)	TMRmin (mlO ₂ g ⁻¹ h ⁻¹)	Q_{10} for TMR>8 at $T_b > 8^\circ\text{C}$	TMRexpo at 36.5° C (mlO ₂ g ⁻¹ h ⁻¹)	TMRexpo as % of BMR
Hibernation						
<i>Glis glis</i> ^a	138	0.657	0.015	2.35	0.157	23.9
<i>S. lateralis</i> ^b	280	0.620	0.022	2.11	0.247	39.8
<i>S. lateralis</i> ^b	240	0.650	0.012	2.63	0.245	37.7
<i>S. lateralis</i> ^c	241	0.846	0.017	3.41	0.846	100.0
<i>Spermophilus</i> <i>parryii</i> ^d	1000	0.490	0.012	2.45	0.125	25.5
<i>Marmota</i> <i>marmota</i> ^e	3090	0.190	0.014	2.40	0.084	44.0
Daily Torpor						
<i>Cercartetus</i> <i>nanus</i> ^f	30	0.650	0.022	3.30	0.340	52.3
<i>Glis glis</i> ^a	138	0.657	0.033	2.45	0.358	54.5

^a Data from this study; ^b Heldmaier et al., 1993; ^c Zimmer and Milsom, 2001; ^d Buck and Barnes, 2000 (BMR pers. comm. B. Barnes); ^e Ortmann and Heldmaier, 2000; ^f Song et al., 1997 (includes data from hibernation as well as torpor).

maintained, (2) a thermoregulatory increase of TMR at the lower end of the hibernation temperature range, and (3) hibernation at elevated T_b where metabolic rate depends upon temperature effects. The latter state can be used to extrapolate TMR to the euthermic level of T_b in order to separate the temperature effect from the effect of active metabolic inhibition in hibernation. The extrapolation reveals a theoretical TMR of $0.157 \text{ mlO}_2 \text{ g}^{-1} \text{ h}^{-1}$ at 36.5° C , which is 24% of their BMR ($0.657 \text{ mlO}_2 \text{ g}^{-1} \text{ h}^{-1}$). It estimates that 76% of metabolic depression was achieved by metabolic inhibition and 24% was due to thermodynamic effects of low T_b in hibernating dormice.

Similar estimates have been made for three other species of hibernators, too, supporting the view of a combined action of active metabolic depression and thermodynamic effects (Table 1). The only exception from this was found in *Spermophilus lateralis*, where metabolic rate reduction could be ascribed entirely to the effects of low T_b (Zimmer and Milsom, 2001). The reason for this discrepancy is not clear. Their experimental procedures differed from other studies since squirrels were implanted with electrodes and metabolic rate was measured in a closed system, which required handling of the system in deep hibernation and may have elevated TMR of squirrels. Results from this study revealed a relatively high Q_{10} of 3.4 for temperature relations of TMR, whereas in all other studies the Q_{10} was in the normal range of about 2.4.

The depression of metabolic rate in hibernation is inversely related with body size. In small mammals of about 10 g body mass, TMR may be lowered to $1/100^{\text{th}}$ of BMR, whereas in 5 kg marmots TMR is about $1/20^{\text{th}}$ of BMR (Kayser, 1961; Geiser, 1988; Heldmaier and Ruf, 1992). This calculates Q_{10} effects for BMR and TMR of about 6 in very small hibernators and 2–3 in larger hibernators, suggesting that in very small hibernators, metabolic inhibition of TMR may be more pronounced than in larger hibernators (Geiser, 1988). If this assumption is correct one would expect that the extent of metabolic inhibition, as calculated in Table 1, is inversely related with body size. Dormice are the smallest mammals studied so far (Table 1) and indeed revealed the highest percentage of active metabolic suppression in hibernation. However, a similar extent of metabolic inhibition was also observed in the larger sized arctic ground squirrels. At the present state the number and mass range of species studied is probably too small to draw any conclusions about the comparative physiology of metabolic depression.

Body Temperature and Metabolic Rate During Daily Torpor

During daily torpor, metabolic rate was lowered to a similar extent as in hibernation (Fig. 1). A closer inspection on a log scale (Fig. 3, insert), however, revealed that TMR in daily torpor on average was about two times the level of hibernation TMR, at the same level of T_b . TMR in daily torpor was highly variable and some dormice even reached values close to hibernation TMR. The different level and high variability of TMR in daily torpor may be due to the different duration of daily torpor bouts (~8 h) and hibernation bouts (> 48 h). During daily torpor, mean values of TMR and T_b were obtained during the last hour of a torpor bout, whereas in hibernation bouts, mean values were obtained from the last 8 through 18 hours prior to an arousal. A previous comparison of TMR and T_b eight hours after entrance into the torpid state revealed similar values for hibernation and daily torpor, indicating that their relation does not differ between daily torpor and hibernation, except differences originating from the short duration of daily torpor bouts (Wilz and Heldmaier, 2000).

TMR in daily torpor correlated with T_b over the entire temperature range from 5 through 30° C, and no range of minimum TMR was detectable as observed in hibernation. A regression calculated for TMR and T_b revealed a Q_{10} of 2.45, and the regression extrapolates to 0.358 mlO₂ g⁻¹ h⁻¹ at 36.5° C T_b , i.e., 54.5% of BMR. It suggests that in daily torpor 45.5% of metabolic depression is due to a temperature independent inhibition of metabolic rate and 54.5% is due to thermodynamic effects of low T_b . A similar analysis in the marsupial *Cercartetus nanus* provided similar values of 47.7% for metabolic inhibition and 52.3% for temperature effects (Song et al., 1997).

How to Enter Torpid States

The present analysis of metabolic depression suggests a combined action of temperature-independent metabolic inhibition and thermodynamic effects of low T_b during entrance into torpor. At thermoneutrality torpor can only be initiated by a downregulation of heat production below the level of BMR. In a cold environment this initial metabolic inhibition is much more pronounced because thermoregulatory heat production can be suspended in addition (Fig. 2). Metabolic inhibition creates a deficit of heat generation and T_b decreases. Thus metabolic rate depression is the primer for entrance into torpor. If entrance into torpor depended solely and entirely upon this temperature-independent metabolic inhibition, one would expect metabolic depression to follow the same time course at thermoneutrality and in the cold. However, this is not the case since metabolic

rate decreases faster and reaches lower values than at thermoneutrality, indicating that active inhibition is augmented by thermodynamic effects of low T_b . The mechanism of this synergistic interaction between metabolic inhibition and low T_b is not known. We can assume that metabolic processes contributing to BMR in mammals are tuned for 37 °C T_b and can only be varied to a small extent, like the circadian variations of BMR by 20–30%. Reductions of basal obligatory heat production in this order of magnitude will be sufficient to cause hypothermia even at thermoneutrality. At low T_b all metabolic processes are slowed down, which offers a greater potential for active metabolic inhibition. Thus, the decrease of T_b opens a thermodynamic window for further active inhibition of metabolic rate. A further decrease in T_b will further open the window, until thermodynamic conditions for maximum inhibition of metabolism are reached.

In deep hibernation several metabolic activities are reduced when compared to euthermic values at the same temperature. These include processes of ATP formation like the decrease in mitochondrial respiration, as well as ATP consuming processes like transcription and translation, suggesting a balanced reduction of metabolic processes in hibernation (for review see Storey, 1997; Carey et al., 2003). In general, the synthesis of proteins and other macromolecules is suppressed in hibernation. At euthermia protein turnover may be accounted to 12–25% of BMR, the turnover of RNA about 1.8%, urea synthesis about 2.3% (Rolfe and Brown, 1997). Theoretically, a suppression of these processes could reduce BMR by about 30%, which would be large enough to prime the onset of torpor. Our knowledge about hibernation is largely based on comparisons of deep hibernation with euthermia, and only little is known about biochemical changes during entrance into hibernation. Transcription and translation are inhibited during entrance into hibernation, but temperatures below 18 °C are required for full inhibition (van Breukelen and Martin, 2001, 2002). In Djungarian hamsters, pyruvate dehydrogenase is inactivated by phosphorylation during entrance into daily torpor in parallel with the suppression of metabolic rate, suggesting that glycolytic inhibition and rerouting of pathways from carbohydrate to lipid utilization occur early during entrance into torpor (Heldmaier et al., 1999). However, to really test this paradigm of a metabolic primer for torpor we need more information on biochemical and physiological changes during entrance into torpor.

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Slow Loss of Protein Integrity During Torpor: A Cause for Arousal?

SANDRA L. MARTIN, TIMOTHY DAHL, AND L. ELAINE EPPERSON

University of Colorado School of Medicine, Department of Cell and Developmental Biology and Molecular Biology Program, Denver, CO, USA

Abstract. Hibernation is an adaptive strategy that is exploited by mammals to conserve energy when food resources are limited in winter. The energy savings achieved by multiday torpor bouts is enormous, but more energy could be saved if the animals did not periodically rewarm. Yet all hibernators do rewarm, indicating that periods of euthermia are required either for survival or for orchestrating the torpid state. We previously suggested that the animals arouse for gene expression, specifically to replenish gene products that are catabolized slowly at the low body temperatures of torpor. Here, new data obtained using a proteomics approach is considered together with hibernation work of the past decade in the context of this hypothesis. Although gene expression is depressed during torpor and reactivated during arousal, this is but one facet of a larger picture that emerges based upon current data from a wide variety of studies, namely that many biochemical and physiological processes resume full activity during each interbout arousal after quiescence during torpor. Although this realization does not answer “Why arouse?”, it reshapes our thinking about hibernation at many levels.

Introduction

Hibernation in mammals is a dynamic process that involves repeated cycling between periods of torpor and arousal (Fig. 1). In torpor, golden-mantled ground squirrels hibernating in our laboratory typically maintain core body temperatures (T_b) near ambient (4°C) for ~ 8 days, whereupon they initiate the arousal

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process. Rewarming is rapid, requiring only ~2 hr to raise T_b to 37° C. This more stereotypical mammalian T_b is maintained in the state of interbout arousal for approximately 10–11 hours, and then the animals re-enter torpor, taking just over 24 hours to reattain their lowest T_b (Fig. 1).

The periodic arousals that punctuate torpor bouts in all mammalian hibernators present a paradox because, although deep hibernators enjoy a 90% energy savings compared to remaining euthermic throughout the winter, 70% of the energy used is consumed by the periodic arousals (Wang, 1979). Given hibernation's overall goal of energy savings, such a large energy expenditure for interbout arousals is counterproductive and thus likely to be crucial for the ability to either: (1) survive torpor, or (2) create and maintain the hibernating phenotype.

Results of earlier studies led us to propose that the phenotype of hibernation is derived from the differential expression of genes that are shared among mammals, including nonhibernators (Srere et al., 1992). If hibernation requires

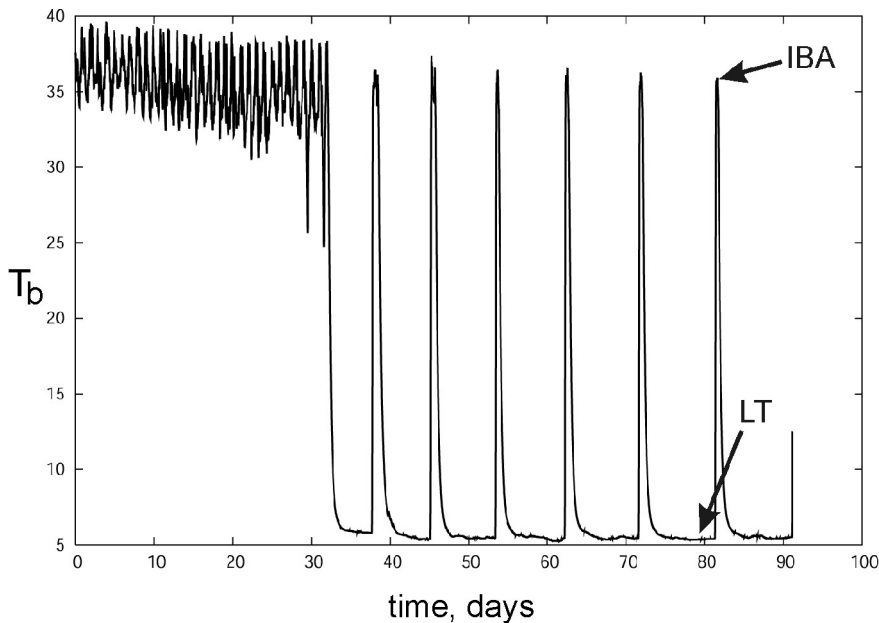


Fig. 1. Dynamic T_b during hibernation in a golden-mantled ground squirrel. Telemetry trace plots T_b every 10 min for 91 days at an ambient temperature of 4° C. Arrows labeled LT (late in torpor) and IBA (interbout arousal) indicate the T_b s used to define these states. SA (summer) is not indicated on the graph because SA animals were killed in July and this trace begins on October 8.

a specific pattern of differential gene expression, then the crucial, differentially expressed gene products must be maintained at levels sufficient to produce the phenotype. The low T_b s of torpor preclude biosynthesis and replenishment of those critical gene products, whilst permitting their slow degradation. In this scenario, an interbout arousal would be required to replenish the essential gene product(s) whose level falls below what is required to maintain the torpid phenotype (Martin et al., 1993). One prediction of this model is that the biosynthetic apparatus would not be capable of mRNA and protein synthesis during torpor when T_b is near 4° C, as during deep hibernation in golden-mantled ground squirrels.

Results of metabolic labeling experiments using radioactive nucleotide and amino acid precursors to study RNA and protein synthesis, respectively, provided early support for a profound reduction in the biosynthesis of both types of gene products during torpor that was reversed during interbout arousal (Bocharova et al., 1992; Gulevsky et al., 1992; Zhegunov et al., 1988). Subsequently, significantly more data have been gathered demonstrating that global mRNA and protein synthesis slow or cease during torpor and that these biosynthetic processes fully reactivate, or even hyperactivate, upon interbout arousal (Frerichs et al., 1998; Knight et al., 2000; van Breukelen and Martin, 2001; van Breukelen and Martin, 2002b).

Recent studies demonstrated that the steady-state levels of most specific mRNAs are unchanged as a function of torpor and arousal (Epperson and Martin, 2002; O'Hara et al., 1999), although there are now numerous examples of specific mRNAs that increase or decrease, usually seasonally, for hibernation (reviewed in Carey et al., 2003). Catabolism of mRNA is apparently slowed during torpor (Epperson and Martin, 2002; Knight et al., 2000), although it does occur based upon examples of specific mRNAs that are increased in winter compared to summer, but more so in interbout arousal (IBA) than in torpor (Andrews et al., 1998; Epperson and Martin, 2002). The steady-state levels of the majority of proteins also appear to remain relatively constant during torpor (Martin et al., 2000), although protein populations have not been examined as often or as thoroughly as mRNAs, probably due to the relatively greater technical difficulties associated with protein work. Here we report the results of a quantitative analysis of changes in the steady-state levels of soluble liver proteins as a function of hibernation using 2D gel electrophoresis. Although the numbers of spots did not differ significantly according to hibernation state, global changes in the saliency associated with each spot were significantly different in a

way that is consistent with a general loss of integrity of proteins during torpor. Biosynthesis of gene products during periodic arousals from torpor is just one of many critical biochemical processes that are fully reactivated in IBA after quiescence during torpor. This realization suggests that the key question to answer regarding hibernation in mammals is not what adaptations of which individual proteins are necessary to improve function in the cold. Rather, the most significant question may be: what adaptations allow the hibernator to cool and slow all processes such that no harm is done either by the slowing or by the hyperactivation that accompanies each interbout arousal?

Materials and Methods

Animals, Tissue, and Protein Preparation

Golden-mantled ground squirrels were obtained and used as described in Epperson and Martin (2002). The liver was snap frozen in liquid nitrogen and stored at -80°C . 200 mg of liver were homogenized in 0.5 M sucrose with protease inhibitors; total protein content in the extract was determined using a BCA Protein Assay Reagent Kit (Pierce). 20 μl aliquots of extract were snap frozen then stored at -80°C .

Two-dimensional Gels

A methanol/chloroform precipitation was used to remove lipids from 150 μg of total liver protein as described in Wessel and Flugge (1984). The pellet was dissolved in sample buffer (Taylor et al., 2000) and used to rehydrate Immobiline DryStrip, pH 3–10 nonlinear (Amersham Pharmacia Biotech) overnight at room temperature under mineral oil. Isoelectric focusing was performed on a Multiphor II apparatus (Amersham Pharmacia Biotech) and the focused strips were applied to a 9–16% SDS-polyacrylamide gradient gel for size separation. To control for environmental effects on 2D gel electrophoresis such as incomplete isoelectric focusing, incomplete polyacrylamide polymerization, buffer variation, temperature variation, diffusion, etc., gels were always run in sets of three with one summer (SA), interbout arousal (IBA), and late in torpor (LT) sample in each set. The gels were stained using SYPRO ruby (BioRad), destained, then scanned on a Typhoon 9400 and analyzed with ImageQuant 5.2 (Molecular Dynamics). The autodetect spot feature of Melanie 4 software (Genebio) was used to define spots, with pixels set to 5, smooth set to 2, a lower limit for saliency of 250. Selected spots were further refined by removal of gel

edge artifacts. The Melanie Spot Report was used to obtain quantitative values for the numbers of spots, and the volume, intensity, and saliency for each spot on each gel. Significance was evaluated using ANOVA, followed by Tukey's post-hoc test to evaluate pairwise differences as indicated. Statistics were done using KaleidaGraph 3.6 (Synergy).

Results

Animals sacrificed in summer (SA), during interbout arousal (IBA), and late in torpor (LT) were chosen for this study. The underlying hypothesis leading to this selection was that proteins particularly important for enhancing the hibernating phenotype will increase their abundance in winter (or decrease in winter if they act to repress or hinder the hibernating phenotype), those not necessary will be decreased, and those with indispensable function will remain constant. This hypothesis predicts that changes in proteins important for hibernation will be shared by the torpid and interbout aroused states and distinct from summer. The hypothesis that loss of gene products by catabolism during torpor necessitates the interbout arousals can also be tested using the same dataset because it makes the prediction that proteins will decrease during torpor and reappear during IBA.

Individual total liver protein samples were separated by isoelectric point in the first dimension and by size in the second dimension. Three representative gels are shown in Fig. 2, each with liver proteins from one of the four animals from the three hibernation states examined. The number of observed protein spots did not differ significantly with the state of the animal used to prepare the

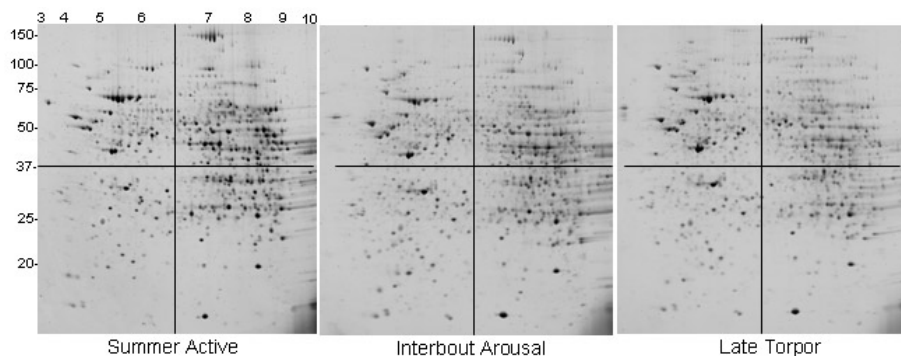


Fig. 2. Two-dimensional gel resolution of soluble liver proteins. The pH range (3–10) and migration of size standards in kDa are indicated. Lines mark boundaries of the quadrants reported in Table 1.

liver extract; however, the greatest numbers of spots were consistently resolved from the SA samples (826), followed by IBA (783) and LT (740), where the numbers in parentheses represent the averages of the four individuals from each state. Although one of our initial goals was to identify the proteins that differed as a function of hibernation state, we found that it was surprisingly difficult to find protein spots that were consistent in all four individuals from one state, yet different from all four individuals in any other state. Of all of the specific protein spots examined, only six differed both consistently and significantly ($p \leq 0.05$) between any two states. The highest levels of all six of these spots were found in the SA samples; five had the pattern $SA > IBA \approx LT$, and one had $SA \approx IBA > LT$ (Epperson 2003). Surprisingly none of the proteins we resolved exhibited the pattern $IBA \approx LT > SA$, which was predicted for proteins that are particularly important for the hibernating phenotype. Although there were several proteins that had increased levels in winter compared to summer, there was sufficient variability among winter individuals to render all of these changes insignificant (data not shown).

The variability in the levels of proteins that appeared to be winter-induced led us to question whether this finding could be explained by the slow loss of proteins to catabolism without resynthesis during torpor. The degree of protein loss during torpor could vary by the length of time that the animal has been torpid since the last interbout arousal, as well as simply among individuals, especially because these are wild animals. During IBA, varying degrees of resynthesis may have occurred, depending upon the length of time at 37° C and/or again depending upon the individual. If proteins are being degraded by general, nonspecific mechanisms as the time in torpor increases, this may be seen on the 2D gels as a decreased fraction of the total protein in specific spots, or as a loss of the sharpness of the protuberance of the spot, i.e., its saliency. Although there were generally fewer spots in winter compared to summer, and specifically in torpid animals vs. IBA, the spot numbers themselves were not significantly different among the different states. However, as is apparent from the data in Fig. 2 and quantified in Table 1, analyses of the protein population from winter animals did indeed reveal reduced saliency in most areas of the gel. It is interesting to note that the larger proteins in the upper half of the gels are more severely affected than the smaller proteins in the lower half, consistent with a stochastic chemical mechanism rather than a specific biological mechanism. We further examined 102 specific spots that were common to all samples to determine whether the same loss of saliency was evident. Again, there is a significant loss

of saliency between summer and both winter samples, with the most severe loss occurring in LT (Table 2). These observations are consistent with the occurrence of slow loss of protein integrity throughout torpor and their replenishment during interbout arousal. The variability among individuals in the two hibernating groups (IBA and LT) compared to summer is likely a reflection of the dynamic nature of the loss and replenishment mechanisms compared to the relatively more stable steady-state levels of the proteins found in the SA animals.

Table 1. Average saliency of liver protein spots from golden-mantled ground squirrels in three states of the annual hibernation cycle. Average saliency values of 2D gel spots were analyzed by quadrant as defined by the lines in Fig. 2. UL, UR, LL, and LR are the upper left, upper right, lower left, and lower right gel quadrants, respectively. Saliency was calculated by Melanie for all spots in each quadrant, with no attempt to correlate spots across gels. These normalized values were analyzed for state-dependent variation by ANOVA; superscripts define groups that differ ($p \leq 0.05$, Tukey). If no superscripts are given, none of the groups differed significantly in that region of the gel ($N = 4$, based upon state of the animal).

State	UL	UR	LL	LR
SA	1828 ^a	1703 ^a	1609 ^a	1773
IBA	1528 ^b	1373 ^b	1432 ^{a, b}	1647
LT	1566 ^b	1336 ^b	1309 ^b	1561

Table 2. Saliency of specific, matched spots. Average relative saliency values of specific, individual 2D gel spots as a function of hibernation state. 102 specific spots were identified by Melanie 4 as present on all 12 gels. The saliency value for each spot was divided by the average saliency value for that same spot on the SA gels ($n = 4$). These normalized values were analyzed for state-dependent variation by ANOVA; superscripts define groups that differ ($p < 0.0001$, Tukey).

State	Normalized saliency	sd
SA	1.15 ^a	0.21
IBA	0.98 ^b	0.17
LT	0.87 ^c	0.16

Discussion

Data from protein analysis by 2D gel electrophoresis demonstrates that the steady-state levels of the majority of abundant soluble liver proteins remained constant as a function of hibernation. Six protein spots decreased significantly during the winter compared to summer, and one decreased in torpor relative to either the SA or the IBA (warm) states. Surprisingly, there were no consistent, significant spot differences where the two winter states differed from SA, as predicted for a protein that is particularly important in determining the hibernating phenotype (Epperson 2003). If such differences do occur during winter, it is possible that they were missed because the protein populations are in a dynamic state during both torpor and IBA. Such an interpretation is consistent with the high degree of variability in the amounts of specific spots seen among the individual extracts from both of the two winter states. Globally, this is evidenced by the loss of saliency. A significant loss of saliency, likely reflecting loss of protein integrity, was observed both when values from all proteins that could be detected were considered, as well as for just those specific proteins that were unambiguously the same protein in all samples. Nonspecific mechanisms of protein damage, such as subtle hydrolysis or modifications, would be expected to more severely affect larger proteins, first by “blurring” the spot, and, as these effects accumulate, result in a loss of intensity of the spot. This type of damage is not expected to lead to the appearance of novel smaller spots, however, because that would require very specific mechanisms and sites of cleavage or modification.

Results of many studies over the past decade demonstrate that gene expression slows or ceases during torpor and is fully restored upon each periodic arousal (reviewed in Carey et al., 2003). The results of this study indicate that some proteins are lost during torpor, likely by a stochastic process (because of the variability among individuals). These findings are consistent with the predictions made by the hypothesis that animals arouse to replenish proteins that are crucial for the hibernating phenotype. Nevertheless, the fact that protein synthesis all but ceases during torpor and is reactivated during IBA does not establish that the purpose of the interbout arousal is for gene expression. In fact, it has become increasingly clear that a wide variety of biochemical and physiological processes slow or cease during torpor, yet are reversed rapidly to typical euthermic status (or greater) during each interbout arousal. These processes include transcription, translation, cell division and migration, mitochondrial respiration, sleep, and immune system function (reviewed in Carey et al., 2003; van Breukelen and Martin, 2002a), as well as synaptic regression with its molecular correlates

(Arendt et al., 2003, and references therein). At this point it is not possible to know which, if any, of these processes are required for survival or for orchestration of the torpid state, or if the key process remains to be discovered. However, these recent observations force the realization that numerous biochemical processes are simply reversibly slowed during torpor then reactivated during each of the many interbout arousals that punctuate the hibernation season. This realization in turn shifts the important questions in hibernation biology away from trying to understand the molecular adaptations that permit improved function in the cold; instead, there is a need to understand how hibernators protect themselves from damage during the transitions between torpor and arousal.

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A Technique for Modelling Thermoregulatory Energy Expenditure in Free-ranging Endotherms

CRAIG K. R. WILLIS,¹ JEFFREY E. LANE,¹ ERIC T. LIKNES,² DAVID L. SWANSON,²
AND R. MARK BRIGHAM¹

¹ University of Regina, Department of Biology, Regina, Saskatchewan, Canada

² University of South Dakota, Vermillion, South Dakota, USA

Abstract. The development of small temperature-sensitive radiotransmitters has allowed researchers to measure skin temperature (T_{sk}) or body temperature (T_b) and quantify temporal patterns of torpor use by free-ranging animals (e.g., number of bouts, length of bouts). However, simply addressing temporal patterns of heterothermy limits the scope of potential research questions because temporal patterns may not correlate with energy savings. Our objective was to devise a predictive model for thermoregulatory energy expenditure, using T_{sk} and ambient temperature (T_a) as independent variables. We used open-flow respirometry and temperature telemetry to quantify the metabolic rate (MR) and T_{sk} of big brown bats (*Eptesicus fuscus*) over a range of T_a from 0° C to 40° C. We calculated regression equations relating T_a , T_{sk} , and MR for each of four different thermoregulatory states: steady-state normothermia, cooling, steady-state torpor, and warming. Our approach may prove useful for quantifying thermal energetics in other free-ranging heterothermic endotherms.

Introduction

Much of our understanding of the energetics of torpor in heterothermic endotherms is based on laboratory research (e.g., Geiser and Brigham, 1999; Hosken, 1997). However, within species, torpor patterns can differ markedly between free-ranging vs. captive (Geiser et al., 2000) or captive-bred (Geiser and Ferguson, 2001) individuals. Field research quantifying torpor patterns in

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free-ranging animals is essential if we are to address questions about the adaptive value of torpor in the wild (Willis and Brigham, 2003). Despite this, comparatively few field studies have addressed the energetics of torpor in free-living endotherms, largely because obtaining real-time measurements of metabolic rate (MR) is logistically difficult for most species in the field (but see Dausmann et al., 2000).

The development of temperature-sensitive radiotransmitters has contributed a great deal to our understanding of temporal patterns of torpor in free-ranging animals. For studies of relatively large or small sedentary animals, transmitters can be surgically implanted to measure body temperature (T_b). For smaller animals, external transmitters must be used to record skin temperature (T_{sk}) because the size and reception range limitations of implanted tags make them impractical (e.g., Hamilton and Barclay, 1994; Barclay et al., 1996; Brigham et al., 2000). Both types of transmitters can provide valuable information about temporal patterns of torpor in free-living animals (Chruszcz and Barclay, 2002; Lausen and Barclay, 2003). For example: What ambient conditions are associated with frequent torpor use? What is the frequency of torpor use at different times of year? However, quantifying temporal patterns of torpor tells us little about the energetic savings associated with heterothermy because, clearly, not all torpor bouts are energetically equivalent. For example, at a given ambient temperature (T_a), a short deep bout of torpor will save a small endotherm much less energy than a shallow bout of longer duration (Willis and Brigham, 2003). In this circumstance, simply quantifying the frequency of torpor use may limit research questions about animals' physiological decisions. What is the level of energy savings associated with torpor use in the wild? How does this level of savings balance against potential costs of torpor use? How do reproductive status and life history traits influence these trade-offs? Without some way to estimate MR in free-ranging animals, we cannot evaluate the real costs and energetic benefits of torpor use under different circumstances.

One approach to addressing this issue is to use laboratory data to model thermoregulatory and basal energy expenditure (hereafter thermal energy expenditure) based on independent variables that can be readily measured in the field (i.e., T_a and T_b or T_{sk}). Such models would need to address several features of normothermy and torpor. Within the thermal neutral zone (TNZ), predicting thermal energy expenditure is simple because model-predicted costs should equal basal metabolic rate (BMR; for a resting, post-absorptive, nongrowing animal). Below the lower critical temperature (T_{lc}) of the TNZ, thermal energy

expenditure, or resting metabolic rate (RMR), scales linearly with T_a and T_b for normothermic animals. During torpor, the logarithm of torpid metabolic rate (TMR) scales linearly with both T_a and T_b . Therefore, for both torpid and normothermic animals, linear regression models could describe these relationships. Any predictive model for the energy savings associated with torpor must also address the fluctuations in energy expenditure that occur during cooling and active and passive warming at the beginning and end of a torpor bout. Recent evidence indicates that free-ranging mammals and birds may rely on ambient heat to arouse from torpor passively (Brigham et al., 2000; Geiser et al., this volume; Körtner and Geiser, 2000; Lovegrove et al., 1999; Willis, 2003). For example, tree cavity-roosting big brown bats, (*Eptesicus fuscus*) and foliage-roosting hoary bats (*Lasiurus cinereus*) are exposed to dramatic diurnal fluctuations in temperature within or at their roost sites and may rely on rising roost temperatures to arouse from morning torpor bouts (Willis, 2003). Geiser and Drury (2003) provided a heat source to torpid dunnarts (*Sminthopsis macroura*) and demonstrated that average energy expenditure during passive arousal was only about 70% of BMR. They also showed that the relationship between T_b and MR during passive warming in thermoconforming animals is nearly identical to the relationship between T_b and MR for thermoconforming animals in steady-state torpor (Geiser and Drury, 2003). Thus, predicting MR associated with arousal from torpor in many free-ranging endotherms may simply be a matter of relying on the relationship between T_{sk} and steady-state TMR.

Our objective was to use open-flow respirometry and temperature telemetry data to devise a predictive model of thermoregulatory energy expenditure for a small heterothermic endotherm, *E. fuscus*. We used T_{sk} and T_a as independent variables because they can be readily measured for free-ranging individuals. We selected T_{sk} , as opposed to T_b , because T_{sk} is much more easily measured in free-ranging bats due to the very small reception ranges of surgically implanted radiotransmitters.

Methods

We captured bats using mistnets in riparian woodlands of southeastern South Dakota, near the town of Vermillion (42° 47' N, 97° 0' W). We performed all trials at the University of South Dakota on five nonreproductive/post-lactating female bats from 2–13 September 2001, and five females that were not palpably pregnant from 1–10 May 2002. All animals were adults. Mean mass was 15.0 ± 1.4 g. Following capture, bats were held in cloth bags, exposed to natural

photoperiod, and provided access to water every few hours. Within one day of capture, a temperature-sensitive radiotransmitter (0.75 g BD-2ATH, Holohil Systems Ltd., Carp ON) was glued to the skin between the scapulae, after trimming a small (0.5–1 cm²) patch of fur. We used a hand-held telemetry receiver (R-1000, Communication Specialists Inc., Orange, CA) and five-element yagi antenna (AF Antronics, Inc. Urbana, IL) to detect transmitter signals during metabolic trials. The relationship between transmitter pulse rate and T_a was calibrated to $\pm 0.5^\circ\text{C}$ in a water bath by the manufacturer and verified prior to experiments. Every two minutes we recorded the time required for a transmitter to emit 11 pulses (i.e., 10 inter-pulse intervals). We later calculated the average inter-pulse interval for each recording and determined T_{sk} from calibration curves provided by the manufacturer.

Food was withheld for 12 hours prior to experiments to ensure bats were post-absorptive during recording. We measured each bat's mass to the nearest 0.01 g using an electronic balance (C305-S, Ohaus, Pine Brook, NJ) immediately before and after metabolic trials and assumed a linear decrease in body mass to calculate mass-specific metabolic rates. We used open flow respirometry to determine MR over a range of T_a between 0° and 40°C . For details of the equipment and protocols used to record BMR, RMR and TMR see Willis (2003). In brief, we recorded oxygen consumption at two to four different test T_a for each of the 10 bats using an oxygen analyser (S-3A, Ametek, Paoli, PA). Bats were exposed to each test T_a for one hour, and the minimum 10-minute average MR recorded during that hour was calculated. We recorded BMR first for each individual at a T_a within the TNZ (30.9 ± 2.4 , range 27.0 – 34°C). For two of the bats we then increased temperatures above 35°C to determine the upper boundary of the TNZ. These individuals were not used to record MR at temperatures below T_{lc} . For the remaining eight bats, we decreased the temperature following BMR recording to obtain RMR and TMR values. Overall, we recorded steady-state MR of bats at roughly 5°C intervals between 0 and 25°C , and BMR at ca. 2°C intervals between 27 and 34°C . Data for all 10 bats were used to calculate mean BMR; two bats remained normothermic below $T_a = 27^\circ\text{C}$ and six bats entered torpor. We also recorded instantaneous metabolic rates concurrent with T_{sk} and T_a during periods of cooling during entry into torpor as well as active and passive warming from torpor. In total we recorded four bouts of passive warming from four bats, four bouts of active warming from three bats, and 23 bouts of cooling from eight bats. Twelve cooling bouts were recorded when

bats entered torpor at low T_a , and 11 were recorded from thermoconforming bats that allowed T_{sk} and MR to decline as soon as T_a was reduced.

Ambient temperature in the metabolic chamber was regulated at $\pm 0.5^\circ \text{C}$ by submerging the chamber in a circulating bath (Model 2095, Forma Scientific, Marietta, OH) filled with ethylene glycol and water. Concurrent with T_{sk} and fractional oxygen concentration (FeO_2) of excurrent air, we measured T_a in the metabolic chamber every two minutes using a copper constantan thermocouple and thermocouple thermometer (Model 8500-40, Cole Parmer).

Model Equations

Values are presented as means ± 1 S.D. All model regression analyses were conducted using Systat (Version 9, SPSS Inc.). Non-normal data were log-transformed and significance was assessed at $p < 0.05$. For our predictive model, when ambient temperature was greater than T_{lc} we assumed $\text{MR} = \text{BMR}$. Below T_{lc} bats were either torpid or normothermic. For normothermic bats we used a General Linear Model (GLM) to calculate the relationship between the independent variables T_a and T_{sk} and the dependant variable RMR. During steady-state torpor, T_a and T_{sk} were highly correlated (Pearson $r = 0.99$), so we eliminated T_{sk} from the analysis. We used linear regression to quantify the relationship between T_a and the logarithm of TMR because T_{sk} was a linear function of T_a (see below). We identified individual bouts of cooling and warming by inspecting each bat's time course of MR and T_{sk} for each recording trial. Cooling bouts were divided into two categories: (1) those during which T_a was constant (i.e., cold) when bats entered torpor; and (2) those during which T_a was declining and T_{sk} and MR of thermoconforming bats decreased simultaneously with T_a . Similarly, warming bouts were divided into (1) those in which warming was spontaneous with T_a remaining constant during the warm-up period (i.e., active warming); and (2) those in which T_a was increasing during the warm-up period. The latter category of warming bouts occurred because for some trials we increased the temperature in the metabolic chamber at the end of a recording session while still recording MR and T_{sk} . For entry into torpor, we calculated total energy expenditure for each cooling bout. We used GLM to quantify the relationship between energy expenditure for the bout and five independent variables: Bout duration (Dur, minutes), starting T_a (T_a), change in T_a during the bout (ΔT_a), starting T_{sk} (T_{sk}), and change in T_{sk} during the bout (ΔT_{sk}). For the analysis of cooling bouts at a constant T_a , ΔT_a was not included. We were not able to use GLM to model energy expenditure associated with warming bouts because of a small sample size.

For passive warming bouts we relied on our model equation for TMR (Geiser and Drury, 2003; see above). To predict the cost of active warming we calculated the average metabolic rate for all the warming bouts and used this value in our calculation of daily energy expenditure.

To demonstrate the applicability of the model to field data, we used it to predict daily energy expenditure values based on roost T_a and T_{sk} for a hypothetical free-ranging bat. Roost T_a was based on a typical temperature time course recorded from an *E. fuscus* roost tree in the Cypress Hills of Saskatchewan, Canada. Similarly, T_{sk} was based on a typical time course recorded from bats in the Cypress Hills (Fig. 1). The hypothetical T_{sk} time course included a deep bout of early morning torpor and an early evening shallow torpor bout. For comparison we also calculated the predicted energetic expenditure for a hypothetical bat that remained normothermic at $T_{sk} = 33^\circ\text{C}$ for the entire 24-hour period. We used a conversion factor of 20.083 J per mL O_2 .

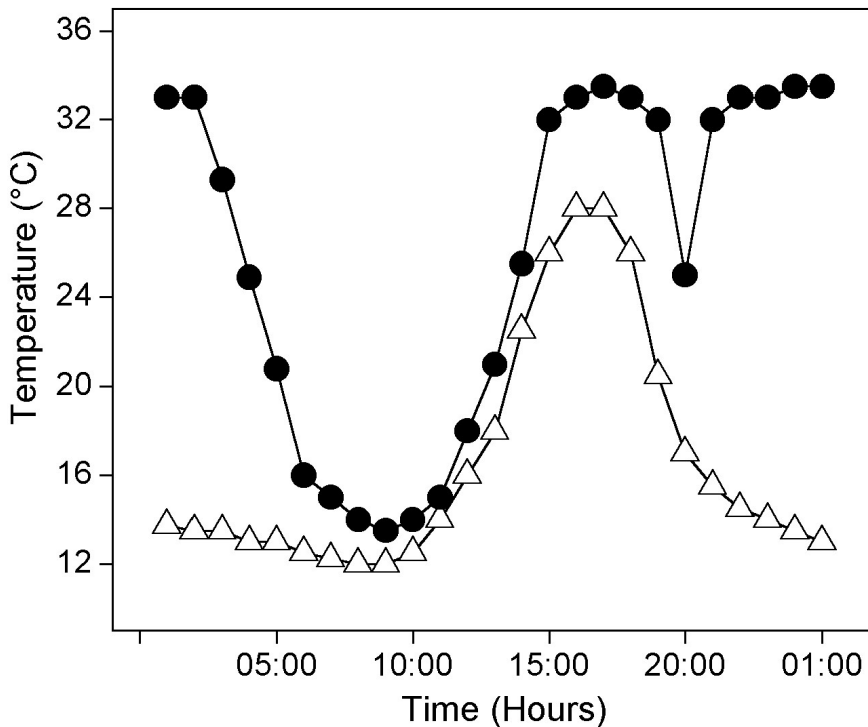


Fig. 1. Time course of T_a (open triangles) and T_{sk} (closed circles) used to calculate daily energy expenditure for a hypothetical free-ranging bat.

Results

As reported elsewhere, mass-specific BMR for *E. fuscus* is $1.14 \text{ ml O}_2 \text{ g}^{-1} \text{ hr}^{-1}$ (Willis, 2003). Despite a small sample size ($n = 6$ datapoints from four bats), the general linear model predicting the RMR of normothermic bats below the T_{lc} of 27° C was significant ($r^2 = 0.98$, $F_{2,2} = 52.1$, $p = 0.019$), and the model equation for these bats was:

$$\text{Mass-Specific RMR} = (0.105 \pm 0.05)T_{sk} - (0.038 \pm 0.005)T_a - (2.22 \pm 1.72) \quad (1)$$

During steady-state torpor, above $T_a = 0^\circ \text{ C}$, there was a significant linear relationship between T_{sk} and T_a ($r^2 = 0.98$, $F_{1,9} = 225.9$, $p < 0.001$), so we only used T_a as an independent variable to describe TMR. There was a significant linear relationship between the logarithm of TMR and T_a ($r^2 = 0.92$, $F_{1,9} = 96.5$, $p < 0.001$). The TMR model equation was:

$$\text{Log Mass-specific TMR} = (0.062 \pm 0.006)T_a - (1.73 \pm 0.096) \quad (2)$$

During constant T_a cooling during entry into torpor, the general linear model including Dur , T_a , T_{sk} , and $^AT_{sk}$ as independent variables was significant ($r^2 = 0.92$, $F_{4,7} = 18.8$, $p = 0.001$). The constant T_a cooling model equation was:

$$\begin{aligned} \text{Mass-specific Energy Expenditure} = & (0.010 \pm 0.004)\text{Dur} - (0.063 \pm 0.022)T_a \\ & + (0.075 \pm 0.020)T_{sk} - (0.053 \pm 0.027)^AT_{sk} - (0.648 \pm 0.145) \end{aligned} \quad (3)$$

For bouts of cooling when the T_{sk} of thermoconforming bats decreased concurrently with T_a , the general linear model was also significant ($r^2 = 0.91$, $F_{5,5} = 9.6$, $p = 0.01$). In this case the model included the variables above as well as AT_a and the equation was:

$$\begin{aligned} \text{Mass-specific Energy Expenditure} = & (-0.008 \pm 0.003)\text{Dur} + (0.086 \pm \\ & 0.019)T_a - (0.028 \pm 0.010)^AT_a - (0.022 \pm 0.014)T_{sk} - (0.043 \pm 0.021)^AT_{sk} \\ & - (0.465 \pm 0.160) \end{aligned} \quad (4)$$

Due to a small sample size of warming bouts, we were unable to devise a predictive model for energetic costs of arousal from torpor. However, we did calculate metabolic rates and estimate energetic costs during passive and active re-warming (Table 1). The average MR of bats was 1.04 times BMR during passive

Table 1. Average metabolic rates (MR) recorded during bouts of active ($n = 4$) and passive ($n = 4$) rewarming from torpor, as well as the warming bout duration (Dur), starting T_a (T_a), change in T_a (ΔT_a), starting T_{sk} (T_{sk}), and change in T_{sk} (ΔT_{sk}).

	MR ($\text{mL O}_2 \text{ g}^{-1} \text{ hr}^{-1}$)	Dur (min)	T_a ($^{\circ} \text{C}$)	ΔT_a ($^{\circ} \text{C}$)	T_{sk} ($^{\circ} \text{C}$)	ΔT_{sk} ($^{\circ} \text{C}$)
Passive	1.86 ± 1.91	34 ± 20	14.3 ± 8.6	17.1 ± 9.1	13.3 ± 7.5	14.1 ± 5.8
Active	9.43 ± 2.68	22 ± 10	4.4 ± 2.1	0	7.5 ± 4.8	19.5 ± 5.8

warming and 8.27 times BMR during active warming. We used the average active warming MR to predict energy expenditure during active warming for our hypothetical bat example (see below). For passive warming we used our equation relating TMR and T_a .

Combining these equations and based on values from Fig. 1, we calculated a time course of energy expenditure for each hour of a 24-hour period (Fig. 2) and used these values to calculate daily energy expenditure. The predicted daily energy expenditure was 22.8 kJ/day for a 21 g bat that employed two bouts of torpor, as shown in Fig. 1. The predicted daily energy expenditure for a 21 g bat defending T_{sk} at 33°C over the same 24 hour T_a profile was much greater at 47 kJ/day (Fig. 2).

Discussion

Our predictive model provides a reasonable estimate of the costs of thermoregulation and basal metabolism based on T_a and T_{sk} , independent variables that can be readily measured for free-ranging animals. Using doubly labelled water (DLW) to quantify field metabolic rate, Kurta et al. (1990) reported daily energy expenditures of up to 70 kJ/day for free-ranging big brown bats, much higher than the values we calculated. This makes sense in light of energetic costs we did not consider (e.g., foraging and flight, digestion, reproduction) but indicates that T_{sk} and T_a can be used to calculate accurate estimates of thermal energy expenditure for free-ranging animals. Future studies employing DLW both in the lab and the field are important to validating the accuracy of this type of model.

Predicted energetic expenditure was nearly twice as high for our hypothetical bat defending T_{sk} at 33°C compared to energy expenditure if the bat used torpor. This difference illustrates one potential application of our modeling approach. Previous studies have addressed temporal patterns of torpor use in free-ranging animals (e.g., Brigham et al., 2000; Chruszcz and Barclay, 2002; Lausen

and Barclay, 2003) but none have quantified the energetic implications of torpor use or avoidance on a given day. Addressing detailed questions about costs and benefits of torpor under different circumstance requires that we estimate energetic benefits associated with different depths and durations of torpor. For example, defending a high T_{sk} was obviously costly for our hypothetical bat. However, if the bat was pregnant or lactating, this energetic cost could be balanced by the potential selective benefit of an increased offspring growth rate. Testing this hypothesis also requires specific information about selective benefits of rapid offspring growth, but quantifying energy expenditure is a necessary first step.

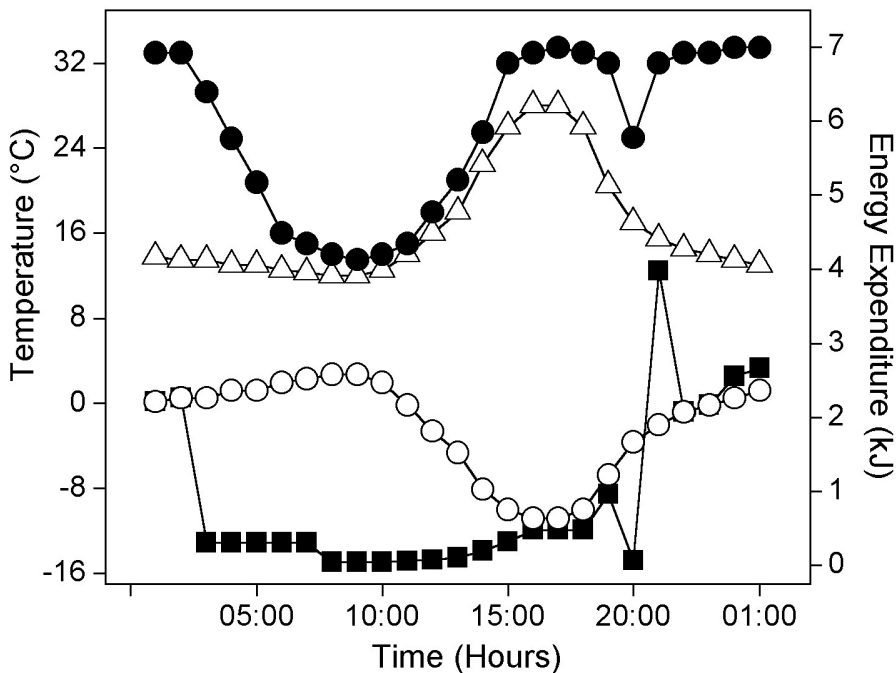


Fig. 2. Time course of T_a and T_{sk} , as shown in Fig. 1, along with two time courses of predicted energy expenditure. Open circles represent model-predicted values for energy expenditure calculated for a hypothetical bat that remained normothermic for the entire 24-hour period at the T_a shown. Closed squares represent model-predicted values calculated for the same bat but based on both the T_a and T_{sk} time courses shown. For the purpose of this illustration the energetic cost of the entire cooling bout between 02:00 and 05:00 was divided evenly between each hour.

The most obvious flaw in our model is the prediction of the energetic costs associated with active warming. We have included a crude estimate of warming costs in our calculation of daily energy expenditure, but it is an overestimate because it does not incorporate any variation in T_a or T_{sk} and is an average of active arousals, which included long warming bouts from deep torpor. It would make little sense for the bat in our hypothetical example to employ the shallow evening torpor bout because the costs of active warming from this bout far outweigh the energetic benefits (Fig. 2). A much larger sample size of spontaneous rewarming bouts over a wide range of T_a and T_{sk} is required to model this relationship.

Our approach is clearly not a replacement for techniques such as DLW, which is well-suited to integrating field metabolic rates averaged over a day or several days. However, this type of model may prove useful in quantifying real-time temporal patterns of energy expenditure and savings associated with torpor and other physiological states in heterothermic endotherms.

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Sex Differences in the Response of Torpor to Exogenous Corticosterone During the Onset of the Migratory Season in Rufous Hummingbirds

SARA M. HIEBERT,¹ JOHN C. WINGFIELD,² MARILYN RAMENOFSKY,² LEAH DENI,¹
AND ANTOINETTE GRÄFIN ZU ELZ¹

¹ Swarthmore College, Department of Biology, Swarthmore, Pennsylvania, USA

² University of Washington, Department of Biology, Seattle, Washington, USA

Abstract. This study took advantage of naturally occurring sex differences in the timing of the autumnal southward migration to investigate the effect of corticosterone supplementation on nocturnal torpor in rufous hummingbirds. On the basis of previously documented body mass patterns in rufous hummingbirds before and during migratory readiness, 5 of 6 females (body mass 3.36 ± 0.05 g) were classified as nonmigratory whereas 10 of 10 males (4.16 ± 0.14 g) were classified as migratory. On four consecutive days, in random order, each bird consumed one of four doses of cyclodextrin-complexed corticosterone dissolved in their sole food source, artificial nectar. Nightly torpor duration increased with corticosterone dose ($P < 0.0001$), was inversely related to daily mass gain ($P < 0.0001$), and, for a given dose and daily mass gain, was more sensitive to corticosterone dose in females than in males ($P < 0.006$). These results are congruent with the notion that the torpor of birds in the nonmigratory life history stage is more dependent on current energy status, as indicated by corticosterone levels, than is that of birds in the migratory life history stage.

Introduction

In vertebrates, glucocorticoid hormones are secreted in response to a wide variety of conditions that result in a depletion of energy reserves or increases in “allostatic load” (McEwen and Wingfield 2003). In this context, the general

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function of glucocorticoids is to orchestrate responses that will return the animal to positive energy balance or reduce allostatic load in the induced state. The purpose of our studies on torpor regulation in rufous hummingbirds (*Selasphorus rufus*) is to elucidate the proximate role of glucocorticoids in initiating nocturnal torpor, in an endotherm whose diminutive size and energy-intensive mode of feeding pose frequent energetic challenge. Hummingbirds are one of the most amenable models for studying daily torpor in both daily and seasonal energetic contexts, because torpor is an all-or-none response that varies, at a given ambient temperature, only in duration (Hiebert, 1990). Furthermore, the ability to enter torpor is retained throughout the annual cycle and is not mutually exclusive with reproduction (Calder and Booser, 1973; Hiebert, 1993; cf. Ruby et al., 1993).

Torpor in birds and mammals is not always a response to an immediate "energy emergency" (Hainsworth et al., 1977; Hiebert, 1991, 1993). For example, seasonal heterotherms typically begin their hibernation season with an abundant supply of stored energy. When they begin their period of seasonal torpor, they are responding in a predictive fashion to an energy shortage that will not become evident until the cold weather and food shortages of winter arrive. Their response to this predictable emergency has been shaped by natural selection rather than by proximate indicators of current energy shortage. Likewise, rufous hummingbirds preparing for their autumnal southward migration accumulate fat stores (in the wild: Carpenter et al., 1983; in captivity: Hiebert, 1993) that are sufficient to sustain them for at least two days and nights in captivity without food (unpubl. observation). Once they have begun to fatten, these birds face no immediate energy crisis but consistently enter nocturnal torpor nonetheless (Hiebert, 1993). Each bout of torpor is anticipatory and not a response to current energy status but preparation for departure from the high altitude/high latitude breeding grounds where food sources will become increasingly unpredictable while the cost of flight is certain. However, if glucocorticoid secretion is a response to unexpected disturbances in homeostasis, glucocorticoids may not participate in the regulation of these instances of seasonal or daily torpor when fat stores are ample. The purpose of this study was to compare the role of exogenous corticosterone, the principle glucocorticoid in birds, in regulating the nocturnal torpor of migratory and nonmigratory rufous hummingbirds. In July, adult males in the wild have already begun the southward migration while adult females remain on the northern breeding grounds to incubate eggs and rear the young to fledging. Captive males and females preserve these differences in tim-

ing of migratory readiness, thus making it possible to study conspecifics in both conditions simultaneously. We hypothesize that the nocturnal torpor of males, which have entered the migratory life history stage, should be less responsive to exogenous corticosterone than the nocturnal torpor of females remaining in the breeding or premigratory life history stage.

Methods

Capture, care, and experimentation were approved by the U.S. Department of the Interior, Washington State Department of Fish and Wildlife, and the University of Washington Animal Care and Use Committee. Feeder-baited traps and mist nets were used to capture adult rufous hummingbirds of both sexes on San Juan Island, Washington, USA, in early May. In captivity at the University of Washington, they were maintained in individual cages (76 × 53 × 48 cm) on a photoregime of 14.5L:9.5D. Birds were fed a commercially available artificial nectar (Nekton USA) that is a complete diet providing nutrients, vitamins, and minerals capable of sustaining healthy birds throughout the annual cycle (Hiebert, 1993). Cages were located in a walk-in environmental chamber in which ambient temperature was 20° C during the day and 5° C at night; these temperatures were designed to approximate those experienced throughout the annual cycle by this species (Hiebert, 1992, 1993).

During the experiment, which was conducted in early to mid July when rufous hummingbirds have moved from coastal low-elevation sites to higher altitudes, birds were given *ad libitum* access to artificial nectar from lights-on to 45 minutes before lights-out. The removal of the food at this time provides sufficient time for the gut to empty before lights-out, so that the mass of the bird at lights-out represents an accurate index of the bird's energetic status (Hiebert, 1992). At lights-out, birds were weighed and placed in a smaller cage (18 × 28 × 13 cm) that contained a small perch on which was mounted a fine (40 ga) thermocouple. This thermocouple measured the surface temperature of the bird's abdomen, which accurately reflects the relative core temperature of the bird and provides a temporally precise record of when torpor begins and ends (Hiebert, 1990, 1993). Thermocouple temperature was recorded continuously throughout the night on a Leeds and Northrup Speedomax 250 chart recorder. At lights-on, birds were weighed again and they were returned to their home cages.

To examine the effect of corticosterone on nocturnal torpor, artificial nectar was supplemented with one of four doses of corticosterone complexed with water soluble 2-hydroxypropyl- β -cyclodextrin (0, 0.48, 1.59, and 2.74 μ M

HBC-CORT, Research Biochemicals International). Cyclodextrin (HBC) not complexed with corticosterone was added to the 0, 0.48, and 1.59 μM HBC-CORT solutions so that all four solutions contained the same total concentration of cyclodextrin. We designed our highest dose to equal the dose of HBC-CORT used in a previous study with this species (Hiebert et al., 2000b) so that if we obtained different results in the current experiment we could rule out corticosterone dose as the cause of this variation; in such a case different results would have to be due to sex or seasonal physiological condition, the main focus of this study. This noninvasive method of delivering CORT has previously been shown to result in increased concentrations of CORT in the cloacal fluid as well as in physiological and behavioral responses in rufous hummingbirds (Hiebert et al., 2000a,b). Each dose was offered for one full day on one of four consecutive days, and the order in which the four doses were offered was randomized among the 6 female and 10 male hummingbirds in the study. We chose to test the birds on consecutive days to avoid the possibility that individual birds (primarily females) might change from nonmigratory to migratory condition during the study. The duration of nocturnal torpor was monitored on the night following each day of HBC-CORT treatment.

Rufous hummingbirds are daytime migrants and hence do not exhibit *Zugunruhe*. Instead, birds were classified as being in migratory life history stage or not (hereafter referred to simply as migratory or nonmigratory, respectively) on the basis of percent gain in body mass over the previous six weeks. In a previous long-term study (Hiebert, 1993), the body mass of rufous hummingbirds in autumn migratory condition (4.5 ± 0.3 g) represented an average increase of 20% over body mass in summer nonmigratory condition (3.6 ± 0.3 g). We rounded each bird's percent change in body mass over the previous six-week period to the nearest 10% and classified all birds that had had at least a 20% mass increase during this period as migratory. Birds with smaller percent mass increases were classified as nonmigratory.

Repeated measures analysis of variance (rANOVA) was used to test the effect of HBC-CORT dose and sex on food consumption and daily mass gain in all birds. A repeated measures multiple regression model was then used to test for effects in all birds of the independent variables HBC-CORT dose, food consumption, daily mass gain, and sex on the dependent variable torpor duration. Visual inspection of three-dimensional plots was used to verify that collinearity among the variables HBC-CORT dose, food consumption, and daily mass gain was insufficient to prohibit their inclusion as independent variables in the same

model. Analyses were carried out in Statview 5 (SAS Institute) and checked in Data Desk (Cambridge Software Publishing). Data are presented as mean \pm s.e.m.

Results

Five of six females were judged to be nonmigratory (mean body mass = 3.36 ± 0.05 g, mean mass gain $3 \pm 1\%$, range -1 to 7% , $N = 5$). One female at 4.69 g (mass gain 38%) was classified as migratory. All 10 males were judged to be in migratory condition (mean body mass 4.16 ± 0.15 g; mean mass gain $30 \pm 4\%$, range 17 to 52%) (Fig. 1).

As in earlier studies (Hiebert et al., 2000b), increased HBC-CORT in the present study resulted in decreased food consumption overall (rANOVA, $F_{(3,42)} = 12.20$, $P < 0.0001$), but there was no significant effect of sex on food consumption ($F_{(1,14)} = 1.76$, $P = 0.2$). Increased HBC-CORT also resulted in an overall decreased daily mass gain (rANOVA, $F_{(3,42)} = 9.54$, $P < 0.0001$), but there was also no significant effect overall of sex ($F_{(1,14)} = 0.016$, $P = 0.9$).

When sex was included in the multiple regression model, there was a significant effect of HBC-CORT dose, daily mass gain, and sex on torpor duration (overall $F_{(23,39)} = 5.02$, $R^2 = 0.75$, $P < 0.0001$). Torpor increased as HBC-CORT dose increased ($F_{(3,39)} = 9.00$, $P < 0.0001$), increased as daily mass gain decreased ($F_{(1,39)} = 13.05$, $P = 0.0009$) and, other factors held equal, increased with HBC-CORT dose significantly more in females than in males ($F_{(1,39)} =$

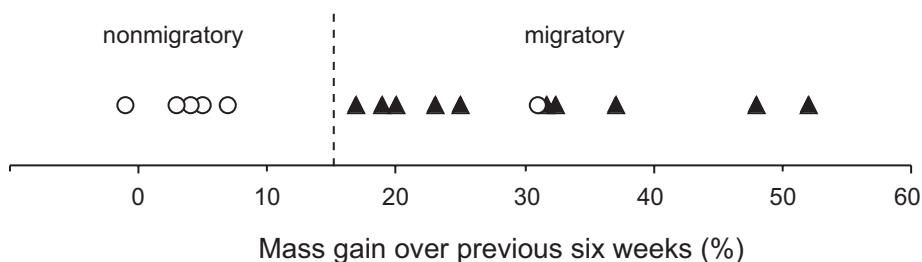


Fig. 1. Classification of male (filled triangles) and female (open circles) rufous hummingbirds (*Selasphorus rufus*) into migratory and nonmigratory condition by % increase in body mass over the six weeks preceding the experiment, which took place in mid-July. Actual % mass gain (shown) was rounded to the nearest 10%; based on the findings of Hiebert (1993), birds with $\geq 20\%$ mass gain were classified as migratory. Sex and migratory condition are strongly associated at this time of year.

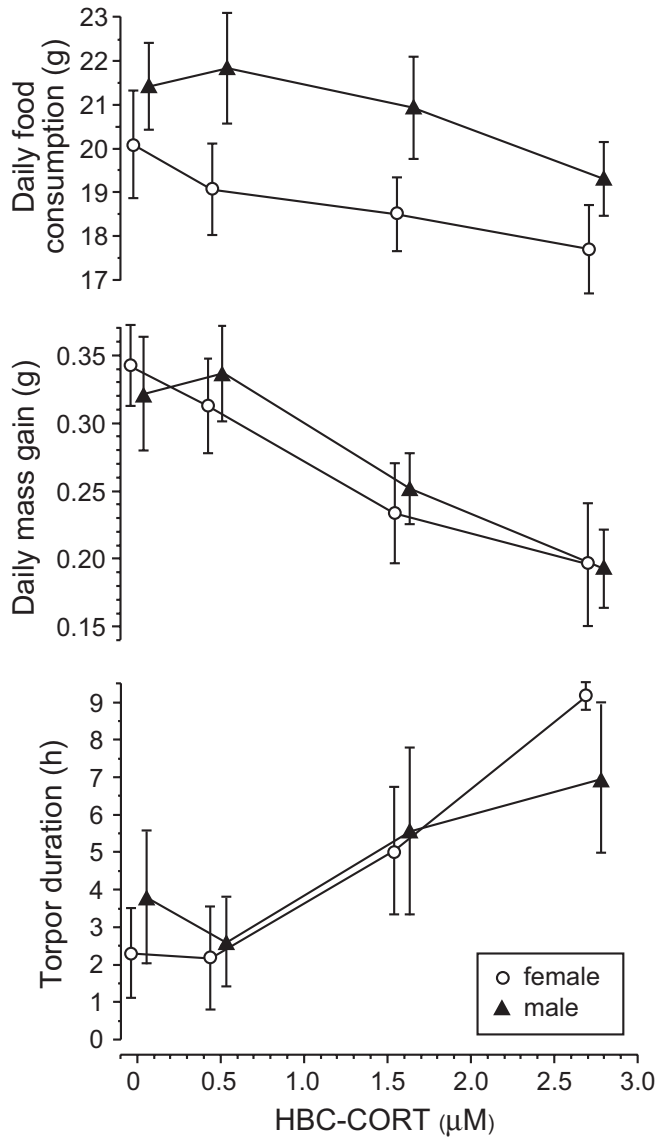


Fig. 2. The effect of exogenous cyclodextrin-complexed corticosterone (HBC-CORT) provided in artificial nectar on food consumption, daily mass gain, and torpor duration in male (\blacktriangle) and female (\circ) rufous hummingbirds (*Selasphorus rufus*). Values for male and female birds were obtained at the same four doses (0, 0.48, 1.59, and 2.74 μM HBC-CORT) but are laterally displaced for visual clarity. Vertical bars represent ± 1 s.e.m.

8.30; $P = 0.006$). There was no significant interaction between dose and sex ($F_{(3,39)} = 0.98$; $P = 0.4$), indicating that the slopes of the torpor response curves were not significantly different between males and females. Qualitatively identical results were obtained in all analyses when sex was replaced with migratory status.

Discussion

During mid to late summer, the duration of torpor in female rufous hummingbirds, judged in this study to be primarily nonmigratory, increased more in response to exogenous corticosterone than did the duration of torpor in males, all of which were judged to be migratory. The results of our study are congruent with the notion that torpor in the nonmigratory life history stage is more dependent on current energy status, as influenced by elevated levels of corticosterone, than is the torpor of birds in the migratory life history stage, which have accumulated a fat store that not only provides energy for flight but also reduces reliance upon immediate resource availability.

Because the migratory timing of males and females is so widely separated in nature, sex and migratory condition on any given date co-vary closely in rufous hummingbirds. We therefore lack the statistical power to separate effects of sex from effects of migratory condition *per se*. However, on the basis of a ten-month study of body mass and torpor in captive male rufous hummingbirds, we believe it is very likely that migratory condition, particularly during the southward migration, has a large impact on energy management and torpor use in this extremely small long-distance migrant (Hiebert, 1993). In these captive males, the autumn migratory season was accompanied both by a sharply delineated increase in body mass (up to nearly double the lean body mass), and by the annually highest duration of torpor, on average nearly double that at any other life history stage (Hiebert, 1993). As a further index of the energetic impact that migration may have on rufous hummingbirds, Calder (1993) estimates that half of each calendar year is occupied by a leapfrog-style migration that requires multiple intermediate stops for the purpose of refattening.

The apparently paradoxical coincidence of the annual cycle's highest body mass and greatest use of torpor during the migratory life history stage contrasts sharply with conditions during the subsequent molting life history stage, when body mass is close to lean mass but torpor occurs rarely, apparently reserved for situations in which energy reserves fall to levels that might threaten immediate survival (Hiebert, 1992). In regulatory terms, the shift to the migratory program

for energy management could thus be accomplished either (1) by increasing the body mass target or setpoint (used here in the broad sense), thus stimulating an increase in energy gain behaviors and a reduction in unnecessary energy expenditures until this new target is reached; or (2) by reducing the sensitivity of the torpor regulatory system to inputs regarding relative energy status, so that torpor is initiated regardless of current energy status. Both mechanisms could result in a reduced sensitivity to exogenous corticosterone, which is hypothesized to act as an index of relative energy status. In the first scenario, the increased difference between a low body mass and a high target or setpoint mass could result in increased endogenous corticosterone production to Level C of Wingfield et al. (1998). Level C concentrations, which correspond to increases beyond normal daily and seasonal fluctuations, would initiate an emergency life history stage that includes, but is not limited to, an increase in the use of torpor. Such a system could result in the observation that even the heaviest wild (L. Carpenter, pers. comm.) and captive (pers. obs.) migratory rufous hummingbirds persist in feeding, even when they are so fat that they are unable to generate sufficient lift to hover steadily at a flower. In this scenario, the torpor regulatory system would remain sensitive to corticosterone within its dynamic range but would not respond to additional exogenous corticosterone because the dynamic range had been exceeded. In the second scenario, exogenous corticosterone would have little effect on torpor because the torpor initiation system is insensitive to the corticosterone signal. This insensitivity could be caused by a variety of factors, such as an increase in corticosteroid binding globulin (CBG; Breuner et al., 2003), a decrease in the number or affinity of glucocorticoid receptors, or a downregulation of other components of the intracellular signaling pathway. In both scenarios, torpor in anticipation of migration would appear to be less facultative with respect to immediate energy balance and corticosterone levels.

The results of the present study cannot distinguish between these two hypothetical possibilities. A previous captive study (Hiebert et al., 2000b) showed that levels of corticosterone in the cloacal fluid of unmanipulated rufous hummingbirds in migratory condition were significantly *lower* than those in mid-day samples (and only marginally greater in evening samples) from unmanipulated molting birds, which rarely entered torpor. These findings, though suggestive, do not conclusively dismiss the possibility that generally increased use of torpor in the migratory life history stage is due to generally elevated corticosterone levels at this time. Likewise, the coincidence of increased torpor use and increased corticosterone levels in captive migratory birds fed diluted artificial nectar

(Hiebert et al., 2000b) provides circumstantial evidence that the torpor of migratory birds may retain sensitivity to corticosterone. Further studies are needed to elucidate the mechanism by which the observed sex difference in the corticosterone sensitivity of torpor is achieved in rufous hummingbirds.

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The Avian Enigma: “Hibernation” by Common Poorwills (*Phalaenoptilus nuttalli*)

CHRISTOPHER P. WOODS AND R. MARK BRIGHAM

University of Regina, Department of Biology, Regina, Saskatchewan, Canada

Abstract. Common Poorwills, small nocturnal insectivorous birds found across western North America, are seemingly unique because of their alleged ability to remain torpid for extended periods during winter. We used temperature-sensitive radio transmitters to assess patterns of torpor use at sites in the Sonoran desert of southern Arizona. Poorwills used torpor extensively whenever ambient temperature (T_a) dropped below 10° C, and there was little evidence for thermoregulation when T_a was above 5° C. During the winter months (December through February), birds remained entirely inactive on 72% of bird-nights, and continuously inactive periods of 10 days or longer were common. The extent of inactivity is similar behaviorally to that of hibernating small mammals. Roost selection, however, facilitated routine passive solar warming, and inactive birds exhibited a regular pattern of arousal on sunny days, followed by reentry into torpor at sunset. We argue that daily arousals are likely an adaptation to the circumstances that characterize surface dormancy. We hypothesize that the relationship between T_a and availability of flying insects at night, in combination with unique ecological aspects of arid regions, contributed to the evolution of multiday torpor use by poorwills.

Introduction

Amongst birds, species in 29 families representing 11 orders have been reported capable of a variety of heterothermic responses (reviewed by McKechnie and Lovegrove 2002). Most birds known to use true torpor are small-bodied specialized foragers, and hummingbirds are the best studied of these (McNab 2002).

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

The other group for whom torpor use is well known are nightjars and their allies, most of whom are nocturnal insectivores (Holyoak, 2001). Most nightjars studied use torpor for brief periods, but among this group and indeed all other birds, the Common Poorwill (*Phalaenoptilus nuttallii*; henceforth poorwill) is unique because of its alleged ability to remain torpid for extended periods e.g., hibernate during the winter (French 1993); although McAtee (1947) summarized unconfirmed reports that some swifts (*Chaetura* sp.) and possibly swallows (*Progne* sp.) may hibernate.

Poorwills are small (ca. 50 g) nocturnal insectivores found across western North America in arid or semiarid habitats with limited overhead cover. Indigenous peoples of the American southwest were ostensibly aware of the poorwill's habits; it is known as Hölchoko, 'the sleeping one' to the Hopi, and Rea (1983) argued that the Pima were aware of its ability to enter torpor. Culbertson (1946) provided the first scientific documentation of torpor and Jaeger (1948, 1949) monitored a seemingly hibernating poorwill. Subsequent workers investigated torpor use by poorwills under a variety of laboratory conditions (Austin and Bradley, 1969; Bartholomew et al., 1957, 1962; Brauner, 1952; Howell and Bartholomew, 1959; Ligon, 1970; Withers, 1977). The development of small radio-transmitters permitted an assessment of torpor use by free-ranging birds, which indicated that torpor is used regularly before and after nesting, as well as in winter (Brigham, 1992; Csada and Brigham, 1994; French, 1993). However, the way in which poorwills use torpor remained unclear. Some authors described torpor bouts in winter with daily or cyclical arousals (French, 1993; Ligon, 1970), whereas others suggested that overwintering birds typically remain continuously torpid for many days (Bartholomew et al., 1957; Jaeger, 1949). We present data on torpor use and "hibernation" by free-ranging birds at an over wintering site, and speculate about why poorwills are the only birds currently known to remain inactive for extended periods during winter.

Data were collected between 1996 and 1999 at two sites in the Sonoran desert ecosystem in southern Arizona (Woods 2002). Both sites were within 100 km of Tucson: the Audubon Society Appleton-Whittell Research Ranch (31° 36' N, 110° 30' W) and foothills of the Tortolita Mountains (32° 32' N, 111° 00' W). Most poorwills were caught at night either using mist nets and taped playbacks of territorial calls or a spotlight and long-handled net (Jackson, 1984; Swenson and Swenson, 1977). Captured birds were outfitted with a temperature-sensitive radio transmitter (Holohil Systems Inc., Carp, ON), affixed to an elastic harness slipped over the wings (Brigham, 1992; Hill et al.,

1999). Transmitters were positioned against the intrascapular skin, where overlying feathers insulated them, and we inferred body temperature (T_b) based on the measured skin temperature (T_{sk} ; Brigham, 1992). Tagged birds were monitored remotely with a Lotek SRX 400 data-logging receiver (Lotek Engineering, Inc., Newmarket, ON). Transmitter pulse rate varied with temperature, and in conjunction with transmitter-specific calibration curves, we were thus able to continuously monitor T_{sk} .

Torpor Use by Poorwills and its Correlates

We conservatively defined torpor bouts based on $T_{sk} < 25^\circ \text{C}$, since one bird was active at 27.3°C and several were active with T_{sk} at or just below 30°C . During torpor bouts, however, T_{sk} dropped rapidly to near T_a , and there was little evidence for thermoregulation unless T_a dropped below about 5.5°C , below which they apparently defended T_b , since T_{sk} did not fall appreciably below 5.5°C regardless of the extent to which T_a did (Woods 2002).

Torpor was used extensively by poorwills whenever T_a approached or dropped below ca. 10°C , and the likelihood that birds used torpor was highly correlated with T_a at sunset ($F_{1,442} = 629.0$, $R^2 = 0.59$, $p < 0.001$, $\beta = -0.77$). Bouts of torpor were recorded as late as 15 June and commenced as early as 5 October (Fig. 1). Entry into torpor by active (versus inactive, see "Inactivity During Winter" below) birds was invariably preceded by foraging. For bouts in October, November, March, and April, torpor bouts commenced 216 min after sunset on average. Bouts by these birds averaged 12.9 h in duration, and the minimum T_{sk} of torpid birds averaged 9.6°C .

We evaluated the influence of prey availability on torpor using artificial lighting to attract and illuminate insects for foraging poorwills during autumn. In each of three open areas used for foraging by a radio-tagged poorwill, we erected a "supplemental" light, which consisted of an 18-watt fluorescent bulb mounted 2 m above the ground. Each light came on at twilight, remained on for four to eight hours, and illuminated an area ca. 20 m in radius. Field observations confirmed that the birds often foraged in the circle of light provided (in fact, at dusk on many evenings birds flew directly to the lights from their day roosts). Torpor was used on fewer nights by birds that had supplemental illumination (34.4% of bird-nights) compared to those that did not (72.3% of bird-nights; $\chi^2_1 = 15.6$, $P < 0.001$). Moreover, other data collected in the context of this study demonstrated that the density of flying insects was tightly and positively correlated with T_a such that when T_a dropped below ca. 10°C few or no flying insects were

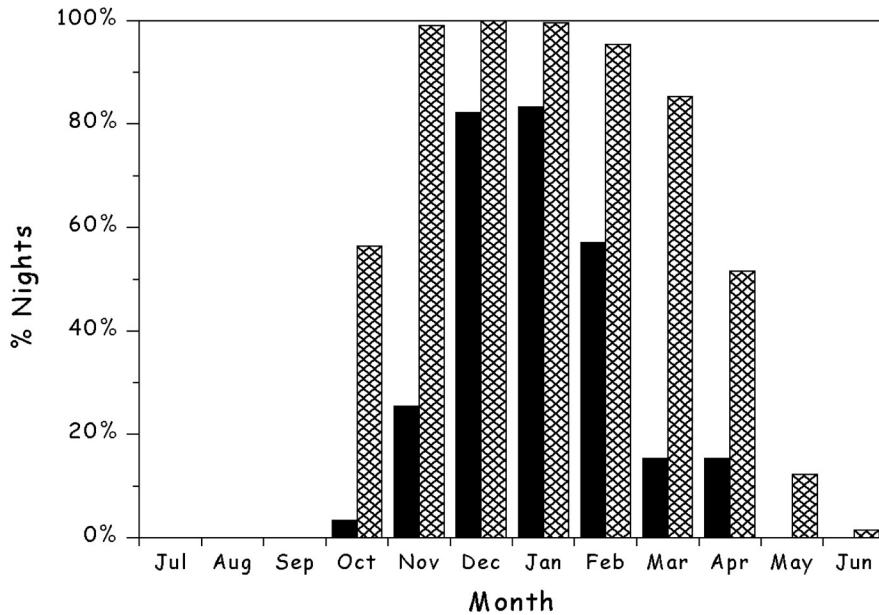


Fig. 1. Percentage of bird-nights by month during which Common Poorwills used torpor (hatched bars) and remained entirely inactive (solid bars) in southern Arizona (see text for distinction between torpor use and inactivity). Sample sizes range from 106 to 270 bird-nights for 8 to 14 individuals per month, and 2,183 bird-nights overall.

available (Woods 2002). Poorwills thus used torpor at times when low T_a increased metabolic costs while foraging became increasingly inefficient owing to a reduction in prey abundance.

Inactivity During Winter, or Do Poorwills Hibernate?

We defined a bird as inactive if it remained in torpor or entered torpor without exhibiting physical movement of any kind at or after sunset on any given evening. The earliest date in autumn that an individual remained inactive was 16 October and the latest in spring was 16 April, but most inactivity occurred during December, January, and February. During those three months, nine individuals were inactive on 469 of 654 bird-nights (72%). Of the nine birds, seven were monitored during the unseasonably warm winter of 1998–9 when daily maximum T_a was ca. 5° C above normal. During the “normal” winter of 1997–8, two birds were inactive on 121 of 132 bird-nights (92%). Overall, individual

nightly inactivity varied between 39.5 and 93.8%. Logistic limitations precluded us from collecting continuous records throughout the winter months for most birds, but nevertheless we recorded continuously inactive periods of ≥ 10 days by seven individuals on 13 occasions, and four periods of inactivity of ≥ 20 days by three birds (maximum 25 days). This provides strong evidence that under natural conditions poorwills remain inactive for considerable periods of time during winter.

So, do poorwills hibernate? Hibernation is defined as the state of winter lethargy with a reduction in T_b and metabolic rate by animals that are homeothermic temperature regulators when active (IUPS Thermal Commission 2001). In a typical mammalian hibernator, individuals spend extended periods (days or weeks) torpid interspersed with short bouts of normothermia during which activity may occur (Geiser and Ruf, 1995). We found the extent to which poorwills remained inactive to be behaviorally similar to hibernating small mammals. The birds we monitored routinely remained inactive for periods of ≥ 10 days, and when not warmed by the sun (see below) they periodically aroused at roughly five-day intervals (Woods, 2002). Moreover, because poorwills remained entirely inactive for long periods, during which time arousals occurred spontaneously (Woods, 2002), we argue that they may make a useful alternative model to test hypotheses about the utility of periodic arousal.

Unlike mammalian hibernators, however, poorwills always roosted in exposed sites that were open to the south or southwest (typically under prickly pear cactus or alongside exposed rocks). Earlier descriptions of poorwill winter roosts also refer to southerly exposure (Jaeger, 1949; Stebbins, 1957; Thorburg, 1953). Winter days were frequently sunny in our study, and because the sun shone directly on the birds, these roosts facilitated routine passive solar warming. Consequently inactive poorwills exhibited a regular pattern of arousal on sunny days, apparently owing to solar warming, followed by re-entry into torpor at sunset (Fig. 2). For inactive birds, T_{sk} rose above 25°C on 84% of bird-days in 1998–9. Torpor bouts by inactive birds averaged 22.7 hours and minimum T_{sk} averaged 6.4°C . It is noteworthy that torpor use, regardless of activity, was ubiquitous through the winter months; poorwills entered torpor on 98.4% of bird-nights between 1 December and 28 February.

Apart from poorwills, no animal of which we are aware remains mostly inactive in exposed situations through the winter, whether shaded or in the sun, although torpid fat-tailed antechinus (*Pseudantechinus macdonnellensis*) regularly bask in the morning and use the sun as an energy source for rewarming (Geiser

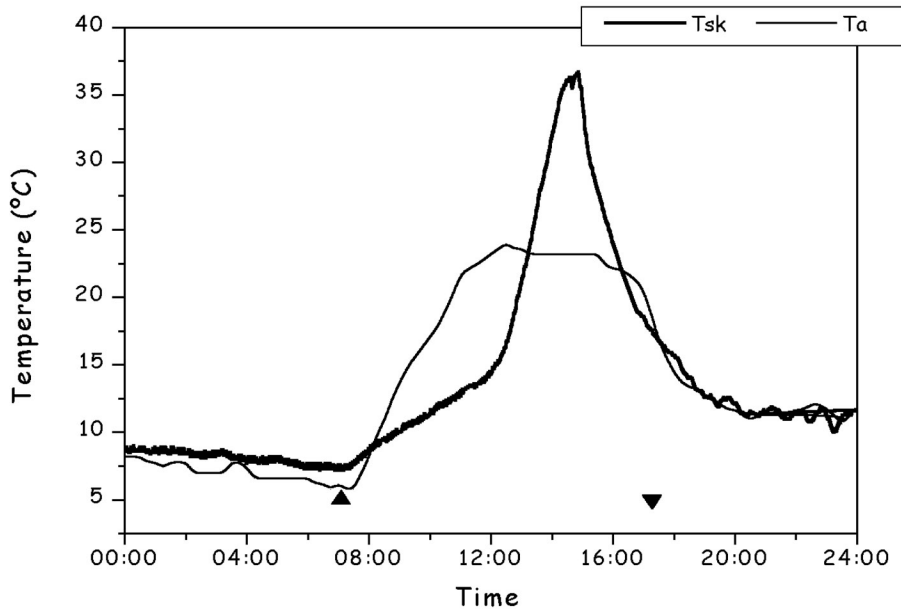


Fig. 2. T_{sk} (thick) and T_a (thin) on 1 Dec 1998 for a Common Poorwill in southern Arizona that remained entirely inactive at sunset. Triangles indicate sunrise and sunset time.

et al., 2002). Without exception, mammalian hibernacula are in sheltered locations. Passive daily arousal in poorwills then is likely an adaptation to the circumstances that characterize surface dormancy. Environmental conditions, microclimate, and sundry other factors are clearly more variable for an exposed animal than one in the protective confines of a burrow or cave. Consequently, poorwills that are passively warmed on a daily basis maintain the ability to become active quickly or to forage at dusk in response to changing circumstances with minimal energetic cost.

The Evolution of Torpor and Inactivity by Poorwills

Factors that limit the range of bats and birds are intriguing because flight allows for the use of spatially discrete habitats. Migration permits breeding and non-breeding requirements to be satisfied in different, often widely separated environments. Perhaps owing to presumed simplicity, factors limiting the range of birds outside the breeding season have been relatively poorly studied. In winter,

torpor reduces the limitations that food availability imposes on where animals can survive, and it is apparently more common in birds than previously thought (McKechnie and Lovegrove, 2002). Why then have no birds other than poorwills birds evolved deep or multiday bouts of torpor? We hypothesize that this physiological capability is an adaptation to a specialized diet of flying insects in an arid environment with limited productivity and was further selected for by interspecific competition with other similarly sized insectivores for prey in winter.

Poorwills have unique physiological adaptations that suggest a long association with hot, arid environments, including a wide thermoneutral zone, an efficient ratio of evaporative water loss to heat production, and white, unmarked eggs (unlike other caprimulgids) with a high solar reflectance (Bartholomew et al., 1962; Lasiewski, 1969; Ingels et al., 1984; Woods, 2002). What makes deserts important for torpor expression? Diapause, torpor, hibernation, migration, and other physiological or behavioral strategies moderate the effects of variation in habitat quality. Moreover, they occur partly in response to increasing variability in components of environmental quality most meaningful to animals living there. Consequently, selection for these strategies should be strongest in habitats where the extremes of variation, as well as their regularity or predictability, occur with the greatest frequency and severity. For example, few animals breeding in tropical regions migrate or hibernate, whereas few breeding in the arctic do not (cf. Newton and Dale, 1996). Arid places with low productivity are characterized by extremes of both temperature and the availability of food and water. These factors often vary daily and/or seasonally in a predictable manner and, consequently, these environments are full of animals that employ hibernation- or estivation-like strategies, which allow residence where conditions may otherwise be unsuitable for survival. Moreover, given the exposed roosts of inactive poorwills and the pattern of torpor bouts, frequent sunny winter days also appear important for overwintering poorwills. Regular solar radiation provides an energy source for passive rewarming not available to heterotherms in regions with long dark winters. Taken together, we propose that the relationship between T_a and the availability of flying insects at night, the variation in these factors both nightly and seasonally in arid regions, the low productivity of these regions, and the frequency of sunny days all selected for the evolution of multiday torpor bouts by poorwills.

Currently, our knowledge about torpor use by other desert-dwelling caprimulgids is limited. Caprimulgids are insectivores whose prey are uncommon during cold weather. Thus all temperate caprimulgids are migratory to

some extent. In North America, migratory poorwills presumably winter within the year-round range of resident birds in the southern United States and Mexico, and also overlap in southern portions of that range with other caprimulgids. Migrants must therefore compete with resident birds for limited food resources in northern portions of their winter range, and with other resident and migratory caprimulgids for those same resources farther south. Further competition may occur with migratory and resident insectivorous owls and bats, and owing to the geography of North and Central America, these animals are concentrated in a region during winter that is geographically much smaller than the region over which they breed.

Perhaps owing to similar foraging strategies, evidence for competitive displacement has been observed within North American caprimulgids and between them and bats (Bjorklund and Bjorklund, 1983/1984; Boyce, 1980; Brigham and Fenton, 1991; Caccamise, 1974; Shields and Bildstein, 1979; Stevenson et al., 1983). Poorwills are the smallest North American caprimulgid, and timid birds as well, and we occasionally observed poorwills leave foraging areas when bats arrived. It is thus plausible that they would be displaced in interactions with larger or more aggressive species, and poorwills probably moderate this competition by wintering north of the winter range of those species, where torpor use is necessary to balance long-term energy budgets. Consequently, historical overwinter survival was probably greater for northern birds that used torpor to lower energy requirements, and extended periods of inactivity likely developed as an extension of daily torpor bouts, as is evidenced by the daily arousal of inactive birds. A similar combination of factors may occur for other caprimulgids overwintering in arid regions, and other "hibernating" species may yet be documented. We propose that Spotted Nightjars in Australia (*Eurostopodus argus*), Band-winged Nightjars (*Caprimulgus longirostris*) in South America, and Freckled Nightjars (*Caprimulgus tristigma*) in southern Africa are candidates worthy of investigation.

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Shivering Thermogenesis in Birds and Mammals

ESA HOHTOLA

Department of Biology, University of Oulu, Oulu, Finland

Abstract. Shivering is the only universal facultative thermogenic mechanism in endothermic vertebrates. Skeletal muscle constitutes a large proportion of body mass and a high scope of metabolic rate, and thus heat production, between resting level and active contraction. Its contractile activity is under rapid and accurate neural control. Together with the inherently low metabolic efficiency of muscle, these “preadaptive” factors have led to an independent evolution of shivering as the main facultative thermogenic effector in birds and mammals as well as other modes of contraction-related muscle thermogenesis in some heterothermic animals. Shivering fulfils the requirements of a facultative mechanism, since it accurately and rapidly tracks variations in thermal conditions and obligatory thermogenesis and is adjusted accordingly to maintain thermal balance. In contrast to the other facultative thermogenic tissue, mammalian brown fat, aerobic muscle has a high inherent capacity for ATP-splitting, which drives the combustion of cellular fuels. Thus, any uncoupling of mitochondrial respiration for thermogenic purposes would incur little selective advantage.

Introduction

Endothermy in birds and mammals, including humans, is based on high basal metabolism and facultative thermogenesis. By definition, facultative thermogenesis is specifically activated in response to cooling during cold exposure (IUPS Thermal Commission, 2003). All biochemical reactions in the cell produce heat as their byproduct (Hochachka, 1974; Block, 1994), and in the resting state at thermoneutrality, these reactions comprise the obligatory basal metabolic rate (BMR). The much higher rate of this basal metabolism forms the basis of

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homeothermy in endotherms (Hulbert and Else, 2000; Ruben, 1995). Its origin is itself an intriguing question, but outside the scope of this overview. When the organism is in a steady state and no external work is done, anabolic and catabolic reactions balance each other, and the amount of heat liberated equals total oxidative metabolism (oxygen consumption). Thus, any reaction has the potential to become a thermogenic effector.

Despite this, only certain reactions were recruited for the specific function of cold-induced thermogenesis during the evolution of endothermy in birds and mammals. This is because there are at least three prerequisites for a reaction to function as a thermogenic effector: (1) It must occur in a sufficiently large tissue mass or be very intense in order to have enough capacity for defending the body against cooling. (2) It must be under instantaneous nervous control so that the level of thermogenesis is accurately set according to the needs imposed by swiftly varying thermal conditions. (3) It must be capable of long-term activation in order to be adaptive during seasonal changes in ambient temperature.

Perhaps not surprisingly, heat production by cold-induced involuntary muscle contraction, known as shivering, was “selected” as the main thermogenic mechanism in both endothermic vertebrate groups, birds and mammals (Hohtola, 2002). Muscle tissue comprises a significant part of their body mass, and the same motoneurons that control normal muscle contraction also form the final commanding pathway for shivering (Hemingway, 1963; Kleinebeckel and Klusmann, 1990). Muscle tissue also has a very large metabolic scope. Its factorial increase from resting to active surpasses that of most other tissues (Clark et al., 2000).

Another indication of the suitability of muscle for thermoregulatory heat production can be found in some heterothermic species. These include brooding python (Hutchison et al., 1966), moths, and honeybees warming up for flight (e.g., Esch et al., 1991), and swimming tunas (Altringham and Block, 1997), all of which use muscular heat production analogous to shivering (i.e. driven by alpha-motoneurons and including muscle contraction) for warming up, or defending against heat loss and thus attain partial endothermy. The other well established thermogenic effector, mammalian brown adipose tissue, fulfils these criteria despite its small mass because it can sustain an intense metabolism and is under sympathetic nervous control (Cannon and Nedergaard, 2004). It is interesting to note that evolution has produced fewer effectors for facultative heat production than for heat dissipation. This probably attests to the higher acute lethality of overheating compared with cooling.

In the following overview, examples and evidence of shivering as a true facultative mechanism that fulfills the criteria listed above are given. Furthermore, evidence for an independent evolution of shivering in mammals and birds is discussed.

Mechanism of Thermogenesis: Simple Principles and Some Misconceptions

The term shivering (cf. *Kältezittern*, *frisson thermique* etc.) is actually rather misleading, since the mechanical consequences of thermoregulatory muscle tone, tremor and microvibrations, which arise from the motor unit contraction cycle, are not prerequisites for thermogenesis and do not add to it in any way. Similar mechanical events accompany all types of static muscle activation, e.g., postural tone. Tremor can actually increase heat loss by increasing convection. The amplitude of tremor and surface vibrations depends very much on the gross anatomy and motor unit function of the species, being invisible in birds but very prominent in some mammals (Kleinebeckel and Klusmann, 1990). Cold acclimation decreases shivering-related tremor in quails (Hohtola and Stevens, 1986). Tremor and microvibrations can be reliable indicators of shivering intensity (May, 2003; Hohtola, unpubl.), but since tremor amplitude depends heavily on motor unit synchronization, it does not always accurately reflect the amount of active motor units (thermogenesis). Although electromyograms (EMGs) also have several limitations in estimating total shivering activity, it is the preferred and most common method to measure shivering (West, 1965; Hohtola, 1982; Tøien, 1992; Hohtola et al., 1998).

Another misleading term, “chemical thermogenesis,” for modes of heat production other than shivering (as if shivering thermogenesis was nonchemical) has been a source of much confusion regarding the simple principles of all cellular thermogenesis. Fortunately, not many textbooks perpetuate this confusing definition any more. All heat emanates, of course, from the chemical combustion of cellular fuels driven by the ATP-consuming cycle at the myofilaments. In a shivering muscle these reactions are exactly the same as during normal isometric muscle contraction. Some of the chemical energy is temporarily transformed into elastic and other types of mechanical potential energy within a muscle cell even when it is contracting isometrically, only to be released as heat when the contraction cycle proceeds. Since no external work is done in shivering, all the chemical energy liberated by cellular combustion is released as heat within the muscle tissue from where it is transported by the convective effect of circulation.

The efficiency of the chemical reactions resulting in muscle contraction is inherently low: only about one fifth of the input energy (in cellular fuels) is converted to external work, even in exercising muscles.

There is also a long-lasting standpoint that shivering is somehow “uncomfortable” and disturbs normal muscle functions (Hochachka and Somero, 2002) and “must” be replaced by some other mechanism during cold acclimation. Although this happens in a number of small mammals, most endothermic vertebrates use shivering thermogenesis even when they are cold-acclimated. It has been repeatedly shown, for example, that winter-acclimatized birds always increase shivering below their lower critical temperature (e.g. Aulie, 1976; Saarela and Hohtola, 2003). Winter-acclimatized arctic fox shiver to increase heat production (Korhonen et al., 1985). Shivering resembles postural activity and interacts with actual muscle contraction with equal flexibility. Firstly, of course, the need for shivering is decreased during activity because of the heat production from the concomitant normal muscle contraction. Secondly, it has been shown both in birds (Nomoto and Nomoto, 1985a; Hohtola et al., 1998) and mammals (Meigal et al., 1998) that shivering can persist during voluntary muscle activity. This is seen as an increased muscle tone in EMGs during a motor task. Furthermore, the increased muscle tone in cold does not affect the accuracy of muscle force control in a biofeedback situation (Meigal et al., 1998).

Interaction of Shivering with Obligatory Thermogenesis

A crucial point for a facultative thermogenic mechanism is that it tracks the rate of heat loss and internal nonthermoregulatory heat production. A vast body of data exists showing that shivering is accurately adjusted to counteract changes in ambient and body temperature (see e.g., Kleinebeckel and Klussmann, 1990). Less data exist, however, on the interaction of shivering with obligatory heat production.

The major components of obligatory thermogenesis besides BMR are motor activity and feeding-related processes (digestion-related thermogenesis, DRT). Facultative heat production should be accurately adjusted according to the changes in these modes of obligatory heat production. Several reports show that the obligatory DRT-related postprandial increase in metabolic rate is absent or lower in cold environments. This suggests that shivering (or NST, in some species) is the facultative component of thermogenesis that is adjusted to a lower level, but this is not usually verified by direct measurements. In pigeons, it was shown that the increase in body temperature from nocturnal to diurnal

levels towards the end of the dark phase incurs an increase in thermogenesis. Electromyographic recordings of shivering show that in fasted or food-restricted pigeons, a higher shivering intensity is needed for this as DRT is less or absent (Rashotte et al., 1999). Furthermore, in a thermoneutral environment, pigeons retain food in the crop and preferably digest it at this time (Laurila et al., 2003). Thus, the heat that would otherwise would have to be dissipated, can be used adaptively.

Although vigorous activity can suppress shivering to some extent via direct neural inhibition (Arnold et al 1986; Nomoto et al., 1985b) the thermal consequences of activity have been shown to modulate the intensity of shivering, and shivering can continue during voluntary muscle activity (Nomoto and Nomoto-Kozawa, 1985b; Hohtola et al., 1998; Meigal et al.; 1998).

Capacity and Endurance

Not all muscle types are suitable for shivering, however. Since the need for thermogenesis can last for hours or even days, only the most aerobic, fatigue-resistant muscles and motor units can be recruited for shivering. In man, it has been shown that the contraction level of a shivering muscle reaches maximally 16–20% of the maximal voluntary contraction (Bell et al., 1992; Haman et al., 2004). This indicates that only a very limited population of muscle fibers are recruited for shivering, these being obviously of the most fatigue-resistant type. Thus shivering can increase the rate of oxygen consumption maximally 4–5-fold, while bouts of running or flying can incur a 20-fold increase. The duration of such bouts is limited to hours, while shivering goes on for days or weeks. In that sense, shivering resembles very much the activity of postural muscles.

The metabolic fuel for shivering is either carbohydrate or lipid (fatty acids), as in normal muscle contraction. Many species differ in the pattern of shivering, showing either a bursting or continuous electrical activity (Hohtola and Stevens, 1986). In man, it has been shown that the pattern of shivering has an influence on the partition of fuels for shivering, the bursting and continuous pattern favoring carbohydrates and lipids, respectively. Cold exposure often causes changes in muscle metabolism that increase aerobic capacity and fatty acid oxidation (see Dawson et al., 1992). In addition, changes in fiber composition (Ballantyne and George, 1978) and mitochondrial biogenesis (Wu et al., 1999) have been observed.

Phylogeny of Shivering

A survey of literature reveals that all mammals and birds that have been appropriately tested show electromyographic signs of shivering when exposed to cold (Kleinebeckel and Klussmann, 1990). By contrast, no such response is seen in reptiles, although the evidence is mostly indirect, i.e., based on measurements of metabolic rate in cold-exposed animals. At least one experiment, however, shows a complete lack of muscle tone during cooling in a lizard (*Tubinambis* sp., Hohtola and Johansen, unpublished).

Phylogenetic evidence shows that mammals and birds evolved from separate reptilian ancestors. Although the possible endothermy of theropod dinosaurs (the ancestor of birds) has been widely discussed, there is no evidence of a common endothermic ancestor for both birds and mammals. Therefore, shivering thermogenesis probably evolved in both groups independently.

Shivering is basically an increase in muscle tone and thus resembles postural activity of skeletal muscles. The differences in the postural system of reptiles and endothermic vertebrates have prompted the suggestion that postural activity formed the neuromuscular substrate that was transformed to shivering (Heath, 1968). Subsequent studies have provided indirect theoretical and experimental evidence for this (Hohtola, 1981; see also Muir, 2000).

Can we find any differences in shivering between birds and mammals, which would support the notion that they originated independently? Since the biochemical reactions producing the heat are certainly identical, eventual differences must be sought in the function of motor units, the basic contractile units of skeletal muscle, and the central nervous system pathways that drive shivering. Motor unit function can be studied by recording the electrical events coupled to muscle contraction. Using such electromyographic methods, the size and synchronization of contracting motor units can be analyzed.

Both in birds and mammals, small motor units are first recruited for shivering. In birds, there is a gradual recruitment of larger units with increasing level of shivering in a smooth “crescendo” pattern (Hohtola, 1982, Tøien, 1992). In mammals, however, the initial recruitment of small units produce a “thermoregulatory muscle tone” that is followed by grouped discharges of motor units (“true shivering”) at higher contraction intensities (Klussmann et al., 1969; Lupandin, 1980). Thus, there seems to be a clear difference of motor unit control during shivering between birds and mammals, which could be used to shed more light on the notion of an independent evolutionary origin.

Another interesting difference in the motor control of shivering exists between birds and mammals: in both groups the intensity of shivering is modulated by the respiratory cycle but in opposite directions. In mammals, shivering is facilitated during inspiration (Kleinebeckel and Klusmann, 1990), but in birds during expiration (Hohtola and Johansen, 1987; Tøien, 1993).

Because of the differential control of motor units and body shape differences, shivering in mammals usually incurs clear visible tremors, while in birds with smooth muscle contraction and compact body form, shivering-related tremors are rarely observed. Birds probably benefit from this as the convective heat loss is smaller with low tremor intensities. Small birds are known to be more cold-resistant than small mammals. In humans, tremors and the basic recruitment of motor units for shivering can influence muscle function in cold. However, by adaptive use of distal and proximal muscles for work and shivering, such problems can be minimized (Meigal et al., 1998).

Muscle NST?

Muscle NST, by definition, would mean thermally driven heat production without contractile activity. There are several studies that report an increase in thermogenesis (oxygen consumption) either without an associated increase in muscle electromyograms (EMGs) or a disparity between the two, even in species that do not have brown adipose tissue (El Halawani et al., 1970; Hohtola et al., 1989; Saarela and Heldmaier, 1987). Although electromyography is the most reliable method for measuring muscle activity related to shivering, the correlation between EMG and thermogenesis is influenced by a host of confounding factors (Tøien, 1992; Hohtola et al., 1998; Marjoniemi and Hohtola, 1999). Different muscles may have different thermal thresholds for shivering (Carey et al., 1989), shivering may be highly localized if a species has only a few very aerobic muscles (Aulie and Tøien, 1988). Thus such disparities should not be taken as evidence of (muscle) NST without careful analysis. To date, the strongest evidence for nonshivering thermogenesis in muscle in true endotherms comes from cold-acclimated juvenile ducks (Duchamp et al., 1999).

The recent discovery of various uncoupling proteins (UCPs) both in mammals and birds has of course precipitated hypotheses on their role in facultative heat production. It now seems clear, however, that they have a role in the regulation of muscle metabolism rather than actual cold-induced thermogenesis (for examples, see Brand, 2000; Talbot et al., 2003). Interestingly, UCP1-ablated

mice acclimate to cold by shivering thermogenesis despite an increase in other types of UCPs (Golozoubova et al., 2001).

That muscle has a very high capacity of heat production without any uncoupling of oxidative phosphorylation is shown by malignant hyperthermia, a pathological condition, where abnormal Ca^{2+} -cycling can induce a lethal hyperthermia. At least one example exists where the contractile activity of muscle has been sacrificed for increased heat production. In swordfish, an anatomically circumscribed endothermy in the eye region is maintained by a specially transformed muscle (Block, 1994) that produces heat by the sarcoplasmic calcium-cycling without contraction but obviously under the control of alpha-motoneurons. It is noteworthy that the modifications that serve heat production in swordfish have resulted in gross anatomical changes, which cannot be seen in true endotherms.

Resting muscle metabolism is known to vary significantly with changes in blood flow. By diverting arterial blood to intramuscular arteries or to connective tissues of the muscle, blood flow can be nutritive or non-nutritive (Clark et al., 2000). This brings about a significant change in muscle metabolism. Whether this could be thermally driven and be rapidly controlled to enable it to act as a facultative thermogenic mechanism is not known (Eldershaw et al., 1997).

It should be once more emphasized that because muscle has an high inherent capacity for ATP-splitting, there is no need for any uncoupling mechanism for heat production (see Hochachka, 1974). Although it could be argued that such a mechanism without muscle contraction would be more adaptive since the tremor and "uncomfortable" muscle tension would be absent, the basic function of muscle, contraction, would be severely compromised. It is difficult to envision such a selective force. Theoretically, this could work if uncoupling could be turned on and off rapidly by some neuronal system, but neither the motor or autonomic system are known to influence the function of muscle UCPs.

From a viewpoint of the history of science, it seems that the search for thermoregulatory thermogenic mechanisms has recently become reductionistic as physiologists want to find specific molecule or reaction that would explain the facultative thermogenesis in muscle (Lowell and Spiegelman, 2000). This may be because of the renowned success in the discovery of the reactions involved in non-shivering thermogenesis in brown adipose tissue (Cannon and Nedergaard, 2004). However, it is well known that temperature regulation and endothermy are largely based on existing physiological functions (Satinoff, 1978). Shivering thermogenesis is an excellent example of this: normal muscle tone has been modified such that it is driven by thermal stimuli.

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The Impact of Social Interactions on Torpor Use in Hummingbirds

DONALD POWERS

Biology Department, George Fox University, Newberg, Oregon, USA

Abstract. Measurements of metabolic rate and fat deposition were made on a three-species hummingbird guild in southeastern Arizona to determine if the energetic advantage gained by a dominant territorial species (*Lampornis clemenciae*) over subordinate competitors (*Archilochus alexandri* and *Eugenes fulgens*) resulted in less frequent use of torpor. Results showed that *L. clemenciae* was able to store enough fat during the day to avoid nocturnal torpor. Restricted access to food limited fat storage in both competitors, resulting in frequent torpor use. Avoidance of torpor by *L. clemenciae* supports the notion that use of nocturnal torpor by hummingbirds comes with a cost, and that the ability to avoid torpor is an important benefit to dominant species.

Introduction

Social interactions between hummingbirds are largely shaped by energetic constraints. Territorial species that defend food resources for their exclusive use do so because the energetic benefit exceeds the energetic cost of territorial defense (Kodric-Brown and Brown, 1978). Conversely, nonterritorial species must balance their energy budgets in the face of higher foraging costs due to active exclusion by territory owners or to the use of poor-quality energy resources (Pimm, 1978). Yet in many hummingbird guilds subordinate species seem to thrive, leading one to question whether territorial foraging or dominant status provide a significant energetic advantage as has been assumed (Krebs and Davies 1978). In an assessment of the costs of competition for nectar, Gill (1978) noted that he was “increasingly impressed with the subtlety of behavioral alternatives used

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daily by nectar-feeding birds to maintain a state of energy balance.” In fact Sandlin (2000b) showed that the use of “complete information” about a food source by a competitor can lead to foraging strategies that reduce the effects of competition.

The actual energetic success of a nonterritorial competitor hummingbird species is in reality hard to measure because individuals are not easily tracked. This inability to track nonterritorial individuals makes it difficult perform the standard time/energy budget studies associated with cost/benefit analysis. Even in cases where total daily energy expenditure of competitor species have been measured using techniques such as doubly labeled water (Powers and Conley, 1994), the inability to partition energy expenditure into specific activity categories complicates our ability to understand energy management by these species.

One way to compare the energetic state of dominant territorial and subordinate competitor species is to examine their tendency to use nocturnal torpor. For hummingbirds the ability to enter torpor is a protection against an “energy emergency” in which daily energy intake is not adequate to meet energy demands (Hainsworth et al., 1977). Hiebert (1992) showed that an “energy emergency” occurred, and torpor initiated by nonmigratory hummingbirds, whenever energy stores dropped below a set threshold level. Previous studies showing torpor use by hummingbirds experiencing thermoregulatory extremes (e.g., Carpenter, 1974) are consistent with this scenario. With these studies in mind I hypothesized that if territorial hummingbirds have an energetic advantage over competitor species, then they would have less need for torpor and use it less frequently.

Inherent in the suggestion that territorial hummingbirds use torpor less frequently than their competitors is the notion that use of torpor has costs and should be avoided if possible. While several studies have suggested potential ecological and physiological costs for the use of torpor by hummingbirds (Calder III and Booser, 1973; Hainsworth et al., 1977; Hiebert, 1990; Hiebert, 1992), no real experimental validation for these potential costs exist. In the following pages I will make further suggestions for why hummingbirds avoid using torpor and will provide evidence that territorial species gain a competitive advantage by being able to maintain normothermy at night.

Study Site and Species

The studies used in this analysis involved a three-species hummingbird guild located in the Chiricahua Mountains of southeastern Arizona. Details of both the

study area and species have been published elsewhere (Pimm et al., 1985; Powers and Conley, 1994; Sandlin, 2000a, 2000b). This system is ideally suited for studying the impact of social interactions on torpor use because the hummingbird species comprising the guild exhibit distinctly different foraging modes and represent a gradient within their dominance hierarchy. The blue-throated hummingbird (*Lampornis clemenciae*; 8.0 g) is an aggressive, dominant species that exhibits classical territorial behavior that results in the exclusion of potential competitors from its food source. The main competitors in this system are the black-chinned hummingbird (*Archilocus alexandri*; 3.5 g) and the magnificent hummingbird (*Eugenes fulgens*; 7.5 g). *Archilocus alexandri* is a primary competitor that acquires most of its energy by intruding on *L. clemenciae* territories. *Eugenes fulgens* is also subordinate to *L. clemenciae*, but uses a trapline foraging strategy (Powers, 1996) that allows it to avoid frequent interaction with *L. clemenciae* (Sandlin, 2000b).

Methods Summary

Hummingbirds were trapped at dusk so that their fat stores were a result of normal daytime activity. Prior to metabolic measurements, birds were fed (except where noted) to simulate the pre-roost meal that is likely part of a hummingbird's nightly routine (Calder III et al., 1990). Nighttime metabolism was measured under temperature conditions that closely approximated the natural environment using open-flow respirometry. Total body fat was measured prior to roosting, at the onset of torpor, or at the end of nighttime (for birds that remained normothermic) using solvent fat extraction. Total body fat at the onset of torpor was assumed to be the torpor threshold. Details of these methods can be found in Powers et al. (2003).

Fat Storage in Territorial vs. Competitor Species

Fat storage data for all species when feeding primarily from feeders containing an energy-rich 1 M sucrose solution (35% sucrose; Baker, 1975) are summarized in Fig. 1. Under these conditions *L. clemenciae* stored significantly more fat during the day than either competitor species, indicating that territoriality did result in an energetic advantage. This energetic advantage was substantial in that total body fat for *L. clemenciae* amounted to twice the measured torpor threshold for body fat in these species (ca. 4% of body mass; Powers et al., 2003), whereas total body fat in both competitors was only slightly above threshold. The high variability in total body fat observed in *E. fulgens* probably corresponds to vari-

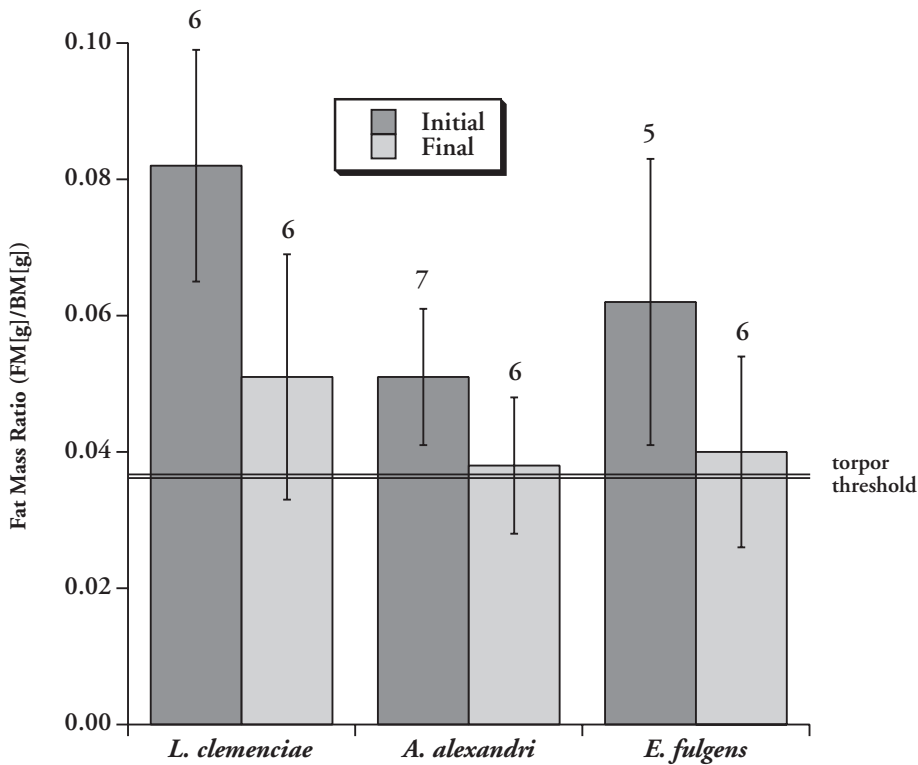


Fig. 1. Ratio of total body fat to wet mass for each study species when fed energy-rich sucrose solution. Data are presented as mean \pm SD. Numbers above error bars are sample sizes. Both initial (pre-roost) and final (onset of torpor or end of night) total body fat in *L. clemenciae* was significantly higher than in the other species.

ability in daily energy intake related to their trapline-foraging behavior. The extra energy stored by *L. clemenciae* more than compensates for the high cost of territorial defense and a daily energy expenditure that exceeds their predicted expenditure by 87% (Powers and Conley, 1994).

Fat storage data for *L. clemenciae* when feeding primarily from feeders containing an energy-poor 0.5 M sucrose solution (17% sucrose) are summarized in Fig. 2. Total body fat was only 72% of that previously measured when energy-rich sucrose solution was used, whereas fat storage in both competitor species was unchanged. Possibly, *L. clemenciae* was unable to increase foraging to compensate for the reduction in energy content of their food due to time

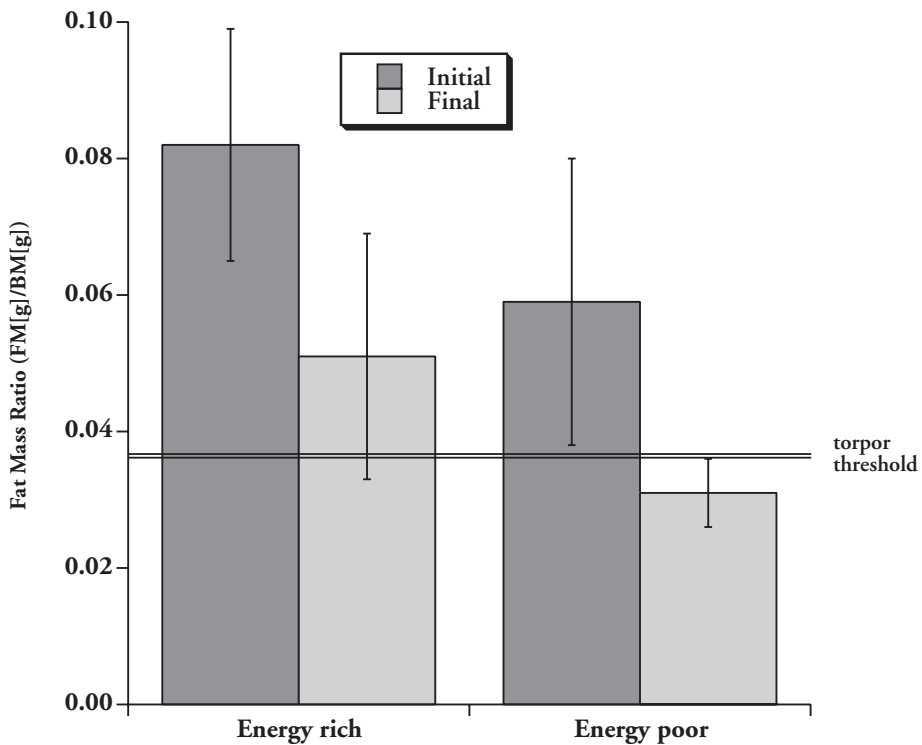


Fig. 2. Ratio of total body fat to wet mass for *L. clemenciae* when fed energy-rich vs. energy-poor sucrose solution. Initial total body fat was lower and final total body fat fell below threshold when the energy-poor sucrose solution was used. In all cases $n = 6$.

requirements for territorial defense or to physiological limits on the ability to process nectar (McWhorter and Martinez del Rio, 1999). In any event, in the face of lower energy rewards, the benefit of being territorial was reduced as has been previously suggested (Kodric-Brown and Brown, 1978).

Relationship Between Torpor Use and Fat Storage

In all species, initial total body fat was significantly greater than final body fat, indicating that fat was catabolized to meet nighttime energy demands. Total body fat in both competitors frequently reached the threshold level at night, causing torpor. The use of torpor by all species is summarized in Fig. 3.

Nearly all *A. alexandri*, who foraged primarily by robbing nectar from *L. clemenciae* territories, used torpor. In this system use of torpor is probably the

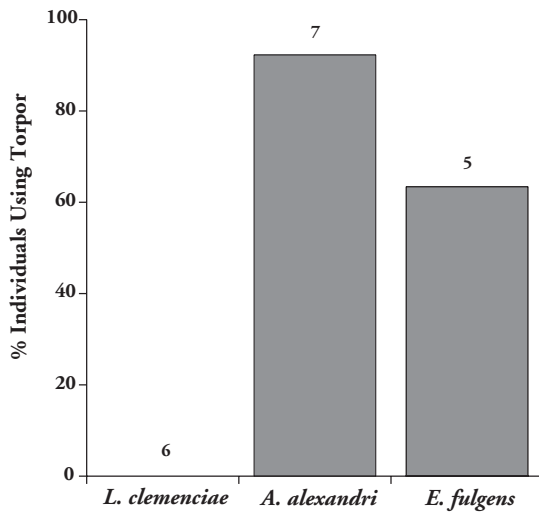


Fig. 3. Percent individuals from each study species using torpor when fed energy-rich sucrose solution.

only way this small, classical competitor can remain in energy balance in light of their low fat storage. Fat storage by *A. alexandri* was not reduced solely by the high energetic cost of being small, but also by competition with *L. clemenciae*. This is supported by the fact that mass-specific daily energy expenditure by *L. clemenciae* is 15% higher than in *A. alexandri*, and that the larger *E. fulgens* also had low fat storage. It would be interesting to look at *A. alexandri* in other systems where they frequently adopt territorial behavior (e.g., Copenhaver and Ewald, 1980), to determine if territoriality would provide sufficient energy to permit nighttime normothermy in spite of the higher metabolic costs associated with small body size.

Lower torpor use and more variable fat storage by *E. fulgens* indicate that for this species traplining has the potential for energy rewards higher than that experienced by *A. alexandri*, but that actual energy intake is unpredictable. Higher energy reward is likely due to their not being constrained by territorial behavior like *A. alexandri* (Sandlin, 2000b), and possible supplementation of their diet with arthropods at a level higher than that which occurs in most hummingbirds (Van Hook et al., unpublished). The end result is that nighttime normothermy can be maintained about 36% of the time.

Final total body fat in *L. clemenciae* reached the torpor threshold prior to the end of night only when feeders contained the energy-poor solution, which was the only time when *L. clemenciae* used torpor (two of six entered torpor). The

fact that no *L. clemenciae* entered torpor when energy was abundant, and only 33% entered torpor when energy availability was reduced, supports the notion that torpor was avoided when energy storage could fuel nighttime normothermy.

Importance of Crop Energy to Nighttime Metabolism and Torpor

Broad-tailed hummingbirds (*Selasphorus platycercus*) engage in hyperphagia to energy load their crop 20 minutes prior to going to roost (Calder III et al., 1990). Is this energy an important supplement to nighttime metabolism? Calder III et al. (1990) showed that *S. platycercus* filled their crops to 179% of predicted volume and suggested that the energy stored was sufficient to support nighttime normothermy without fat catabolism. The use of pre-roost hyperphagia by other hummingbirds has not been studied. Bech et al. (1997) found that a “significant fasting period” (20–100 minutes) was required in two of three species they studied in order to induce torpor. In these species the loss of crop energy may have played some role in the increased incidence of torpor after fasting.

The role crop energy plays in supporting nighttime metabolism was examined in *L. clemenciae* and *A. alexandri* by measuring torpor use in birds denied a pre-roost meal (Powers et al., unpublished; Fig. 4). Torpor use pattern differed only in *L. clemenciae* where the loss of crop energy caused all individuals to use torpor. This use of torpor suggests that in this system dominance and territoriality is not sufficient to support nighttime normothermy exclusively with fat stores.

The most striking result of these experiments was the inability of *A. alexandri* to arouse from torpor. These birds had to be hand warmed and fed at the onset of the active period. If these measurements represent what goes on in wild populations, then the energetic tightrope walked by species like *A. alexandri* becomes narrower, and the importance of their being good competitors amplified (Powers and McKee, 1994; Sandlin, 2000b).

Are There Potential Costs to the Use of Torpor by Hummingbirds?

Because the use of torpor generally results in energetic gain (Hiebert, 1990), the ability of *L. clemenciae* to avoid torpor is advantageous only if the use of torpor has associated costs. The fact that *L. clemenciae* does indeed avoid torpor suggests some benefit to remaining normothermic at night. While several potential costs have been suggested (Hainsworth et al., 1977; Hiebert, 1990; Hiebert,

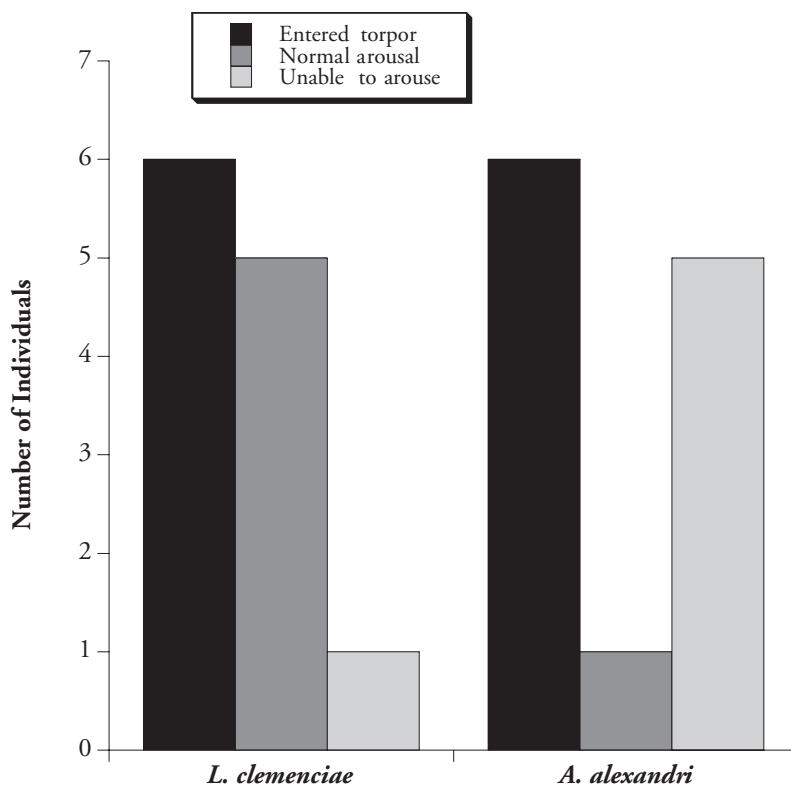


Fig. 4. Number of *L. clemenciae* and *A. alexandri* that used torpor, aroused from torpor normally, and were unable to arouse from torpor when denied a pre-roost meal.

1992), no studies demonstrate that these costs are real. Future studies addressing potential costs of torpor must be done if we are to completely understand the role torpor plays in long-term energy management in hummingbirds.

There are logical reasons why *L. clemenciae* might avoid torpor when they are energetically able. One possibility would be increased risk of predation. There is actually little evidence that adult hummingbirds are a major prey item for any species (Miller and Gass, 1985), and the only information available for the study species is anecdotal at best. However, these hummingbirds likely roost on branches of shrubs or trees, and if they could be located, torpid birds would be unable to escape. There is indication that nocturnal species such as ring-tailed cats (*Bassariscus astutus*) and a variety of arboreal snakes in the study area have to some degree preyed upon hummingbirds (D. Powers, pers. observation). A sec-

ond possible cost is early access to food. At the Southwestern Research Station, *L. clemenciae* typically arrives at feeders about 15 minutes before *A. alexandri* in the morning (D. Powers, pers. observation). While there is no way of knowing if this pattern is related to *A. alexandri*'s regular use of torpor, or perhaps their difficulty in arousing from torpor, the delay in the onset of foraging might put them in the position of having to make up an energy deficit from the very beginning of the day.

Conclusions

Dominant, territorial *L. clemenciae* had a relative body fat content that was twice that measured in its primary competitors, suggesting that an energetic advantage was gained by restricting access to its food source. The higher fat stores of *L. clemenciae* provided sufficient energy to support normothermic nighttime metabolism, whereas both competitor species had to use torpor to balance their energy budget. Energetic constraints were most severe for *A. alexandri*, which needed to use torpor almost every night. The energetic tightrope walked by *A. alexandri* was further evidenced by their inability to arouse from torpor when they entered the nighttime period without energy stored in their crop. The regular use of torpor by both competitor species supports the hypothesis that hummingbirds whose access to energy resources is restricted by social interactions such as territoriality will use torpor more frequently.

Even though the use of torpor results in an energetic savings for hummingbirds, the fact that *L. clemenciae* routinely avoids torpor suggests that torpor has associated costs. If so, then monitoring torpor use in the various hummingbird species in a social group might provide insight into their energetic success. While several potential costs have been proposed both here and elsewhere, none have been experimentally demonstrated for hummingbirds. Before we can fully understand the role torpor plays in the long-term management of hummingbird energetics, studies addressing these potential costs will have to be done.

Acknowledgements

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The Energetics of the Rewarming Phase of Avian Torpor

ANDREW E. McKECHNIE AND BLAIR O. WOLF

Biology Department, University of New Mexico, Albuquerque, NM, USA

Abstract. Avian rewarming rates are related to body mass by $\log R = 0.436 - 0.634 \log M_b$, where R is rewarming rate ($^{\circ}\text{C min}^{-1}$) and M_b is body mass (g). Predicted avian rewarming rates are lower than those for mammals at all but the smallest M_b (< 5 g). The energy required to rewarm from torpor (E_{rewarm}) can be estimated using a simple biophysical model. The model predicts that E_{rewarm} increases linearly with M_b and that the slope of this relationship increases with decreasing torpor body temperature.

Introduction

In the last decade, it has become clear that daily torpor is considerably more common in birds than was previously thought (McKechnie and Lovegrove, 2002). Integrating data on the use of torpor with other aspects of a species' ecology requires an understanding of the energetic benefits and potential ecological costs. One major constraint on the energetic benefits associated with torpor is the energy required to rewarm to normothermic body temperature (Prothero and Jürgens, 1986). Metabolic heat production during rewarming (or arousal) can be considerable. In hummingbirds, for instance, metabolic rates during rewarming from torpor can be comparable to those achieved during hovering flight (Bech et al., in press). In an effort to better understand how biophysical constraints may limit the potential benefits of avian torpor, we investigated the energetics of the rewarming phase. Specifically, we asked two questions. First, how do avian rewarming rates scale with body size, and are they similar to those observed in mammals? Second, how does the cost of rewarming relate to body size and to physiological parameters of torpor such as body temperature?

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Because of the paucity of empirical data, we constructed a model to address the latter question.

Materials and Methods

Rewarming Rates

We obtained rewarming rates for 13 species from the literature (Table 1). When authors did not report rewarming rates, we calculated them from traces of body temperature (T_b). For T_b traces recorded in free-ranging birds, we calculated rewarming rates only when air temperature (T_a) was relatively stable. All body masses (g) and rewarming rates ($^{\circ}\text{C min}^{-1}$) were \log_{10} -transformed prior to analysis by least-squares linear regression.

Energetic Cost of Rewarming

Table 1. Avian rewarming rates.

Species	Body mass (g)	Rewarming rate ($^{\circ}\text{C min}^{-1}$)	Reference
<i>Archilochus alexandri</i>	4.0	1.254	Lasiewski and Lasiewski, 1967
<i>Amazilia versicolor</i>	4.1	0.887	Bech et al., 1997
<i>Eugenes fulgens</i>	6.8	1.121	Lasiewski and Lasiewski, 1967
<i>Melanotrochilus fuscus</i>	7.7	0.457	Bech et al., 1997
<i>Lampornis clemenciae</i>	8.5	0.834	Lasiewski and Lasiewski, 1967
<i>Eupetomena macroura</i>	8.6	0.614	Bech et al., 1997
<i>Patagona gigas</i>	21.0	0.614	Lasiewski et al., 1967
<i>Aeronautes saxatilis</i>	31.0	0.370	Bartholomew et al., 1957
<i>Colius colius</i>	35.1	0.210	McKechnie and Lovegrove, 2001a
<i>Phalaenoptilus nuttalli</i>	40.0	0.429	G. A. Bartholomew and R. C. Lasiewski (unpubl. data, cited in Lasiewski et al., 1967)
<i>Aegotheles cristatus</i>	48.2	0.143	Brigham et al., 2000
<i>Colius striatus</i>	51.0	0.219	McKechnie and Lovegrove, 2001b
<i>Podargus strigoides</i>	452.0	0.055	Körtner et al., 2001

The energy required to rewarm from torpor (E_{rewarm}) comprises two components: first, the energy required to raise the animal's tissues from torpor body temperature (T_{torpor}) to normothermic rest-phase body temperature (T_{norm}), and second,

the energy required to balance the heat lost to the environment during the re-warming process. Hence,

$$E_{\text{rewarm}} = s(T_{\text{norm}} - T_{\text{torpor}})M_b + \int_{t_2}^{t_1} Q \quad (1)$$

where s is the specific heat of animal tissues ($3.43 \text{ J g}^{-1} \text{ }^{\circ}\text{C}^{-1}$), M_b is body mass, Q is the rate of heat loss to the environment, and t_1 and t_2 are times corresponding to the start and end of rewarming respectively. The duration of the rewarming phase (D_{rewarm}) is calculated as

$$D_{\text{rewarm}} = \frac{T_{\text{norm}} - T_{\text{torpor}}}{R} \quad (2)$$

where R is the rewarming rate. To simplify the calculation of the second term of equation (1), we assumed that rewarming occurs linearly, so that

$$E_{\text{rewarm}} = s(T_{\text{norm}} - T_{\text{torpor}})M_b + D_{\text{rewarm}} \left(\text{TMR} + \frac{\text{RMR} - \text{TMR}}{2} \right) \quad (3)$$

where TMR is the metabolic rate during steady-state torpor (i.e., the maintenance phase) and RMR is the normothermic resting metabolic rate.

To generate generalized predictions, we estimated RMR using predicted thermal conductance (Schleucher and Withers, 2001) and predicted basal metabolic rate (BMR; McKechnie and Wolf, 2004). We assumed that $T_{\text{norm}} = 38.5^{\circ}\text{C}$ (Prinzinger et al., 1991), and that $T_a = 0^{\circ}\text{C}$. We used T_{torpor} setpoints that were higher than T_a . Hence, we calculated TMR as $\text{TMR} = \text{TMR}_{\text{Treg}} + C(T_{\text{reg}} - T_a)$, where T_{reg} is the T_a below which a T_{torpor} setpoint is defended (typically $1\text{--}2^{\circ}\text{C}$ below the T_{torpor} setpoint), TMR_{Treg} is the torpor metabolic rate at $T_a = T_{\text{reg}}$ (calculated as a Q_{10} -mediated temperature-dependent decrease in BMR), and C is thermal conductance. We assumed that thermal conductance during torpor was the same as during normothermy. The model is available as a spreadsheet from AEM.

Results

Rewarming Rates

Rewarming rate was strongly and negatively related to M_b (Fig. 1). The slope of this relationship did not change significantly ($t = 0.069$, $df = 21$, $P > 0.05$) when we excluded *P. strigoides*, which is almost nine times heavier than the next

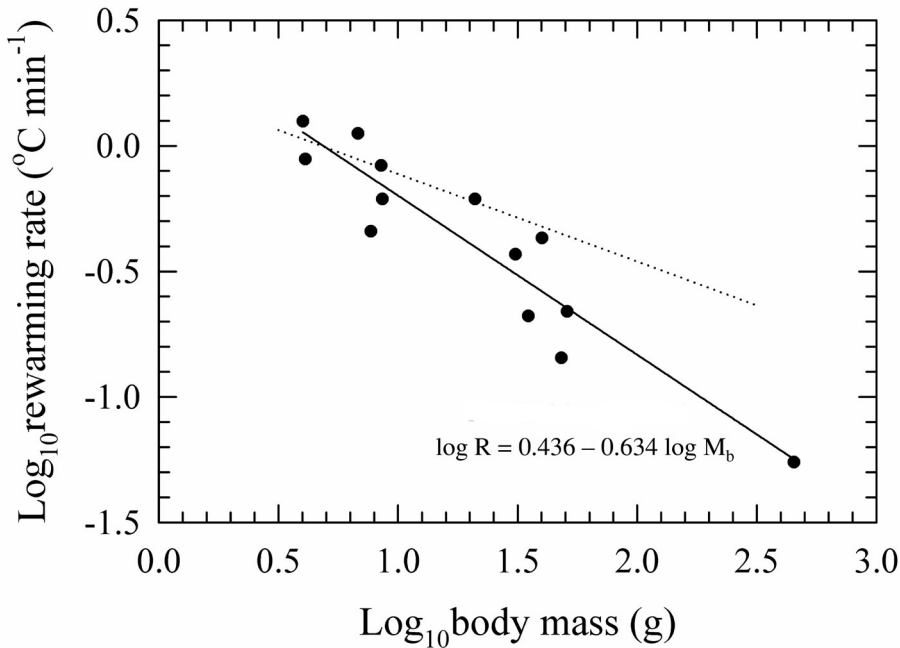


Fig. 1. The relationship between rewarming rate and body mass in birds. The solid line is a least-squares linear regression fitted to the data [$\log R = 0.436 - 0.634 \log M_b$; $r^2 = 0.87$, where R is rewarming rate ($^{\circ}\text{C min}^{-1}$) and M_b is body mass (g)], and the dotted line is the relationship for mammalian daily heterotherms ($\log R = 0.237 - 0.349 \log M_b$; Geiser and Baudinette, 1990).

largest species. In addition, the rewarming rate observed in *P. strigoides* may have been affected by solar radiation (Körtner et al., 2001). The slope of our equation for avian rewarming rates is significantly greater than the slope of Geiser and Baudinette's (1990) equation for mammalian daily heterotherms ($t = 2.811$, $df = 36$, $P < 0.05$).

Energetic Cost of Rewarming

To validate our model for the energetic cost of rewarming, we compared predicted values of E_{rewarm} to those observed in a hummingbird (*Selasphorus rufus*; Fig. 2) and an elephant shrew (*Elephantulus rozeti*; Fig. 2). Our model yielded reasonable predictions of E_{rewarm} in *E. rozeti* at $10^{\circ}\text{C} \leq T_a \leq 25^{\circ}\text{C}$, but underestimated E_{rewarm} at $T_a = 5^{\circ}\text{C}$ (Fig. 2). However, replacing the single allometrically

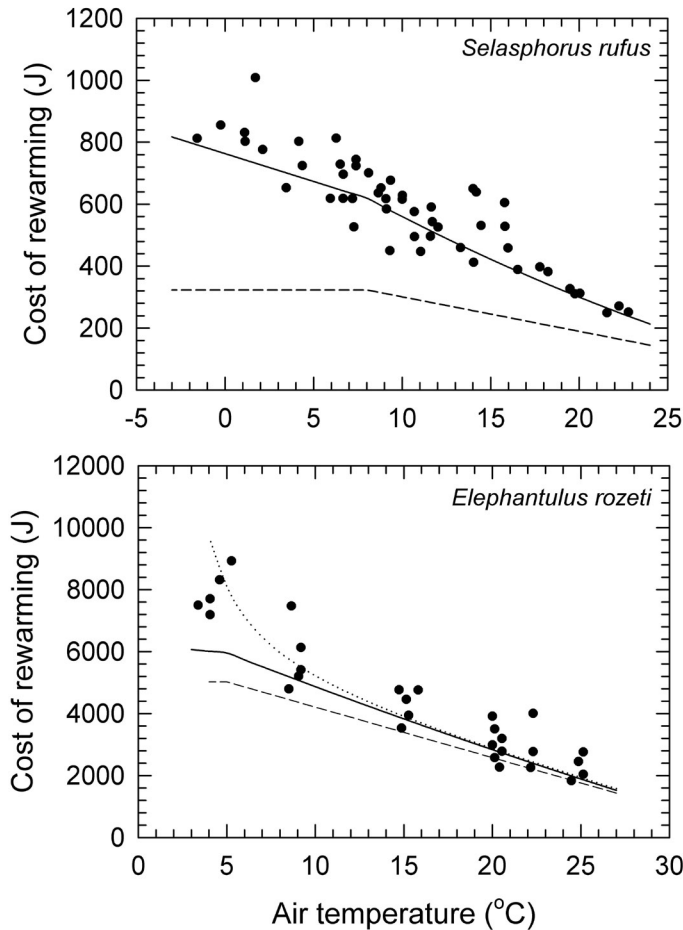


Fig. 2. The energetic cost of rewarming from torpor (E_{rewarm}) in a hummingbird (upper graph; *Selasphorus rufus*; data from Hiebert, 1990) and an elephant shrew (lower graph; *Elephantulus rozeti*; data from Lovegrove et al., 2001). In each graph, the solid line is the relationship between E_{rewarm} and air temperature (T_a) predicted by our model, and the dashed line is the relationship between E_{rewarm} and air temperature predicted by Prothero and Jürgens (1986) and Humphries et al. (2002). The dotted line in the lower graph is predicted E_{rewarm} based on the relationship between rewarming rate and T_a observed by Lovegrove et al. (2001), rather than a single allometrically predicted value.

predicted rewarming rate (Geiser and Baudinette, 1990) with Lovegrove et al.'s (2001) observed relationship between T_a and rewarming rate improved the accuracy of predicted E_{rewarm} at $5^\circ\text{C} \leq T_a \leq 10^\circ\text{C}$ (Fig. 2).

Our model predicts that, for a given T_{torpor} , E_{rewarm} increases linearly with M_b (Fig. 3). The slope of the relationship between E_{rewarm} and M_b is directly related to the extent of body temperature reduction. As T_{torpor} decreases, and hence $T_{\text{norm}} - T_{\text{torpor}}$ increases, E_{rewarm} increases more rapidly with increasing M_b (Fig. 3).

Discussion

Rewarming Rates

Our analysis reveals that the relationship between avian rewarming rate and body size differs from that of mammals (Fig. 1). Since thermal conductance is ca. 35% higher in mammals than in birds (Aschoff, 1981), the differences in rewarming rates likely reflect differences in the rate of heat production, rather

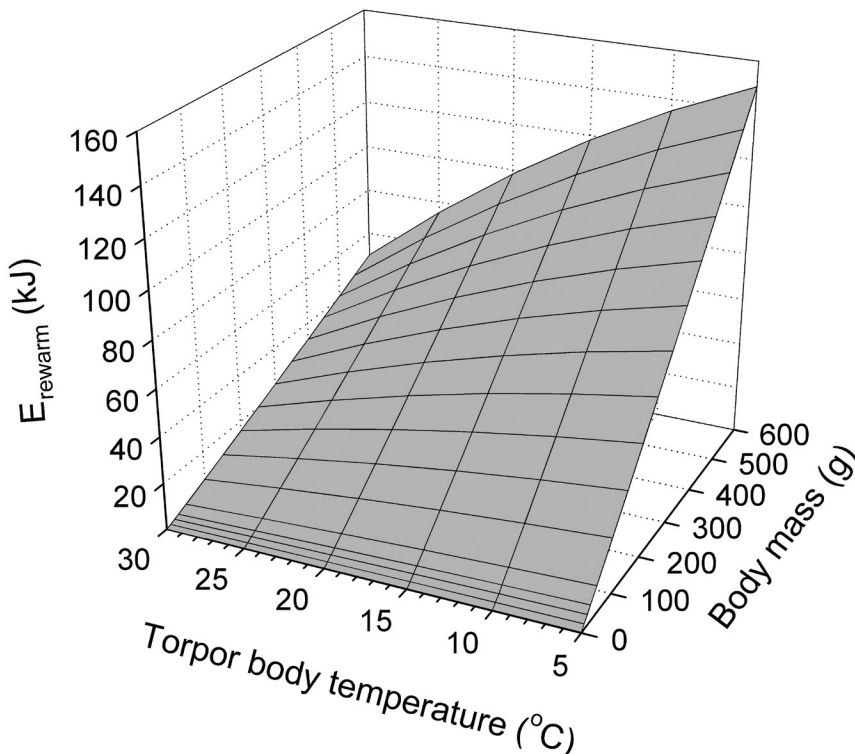


Fig. 3. The predicted relationship for birds between the energetic cost of rewarming (E_{rewarm}), torpor body temperature, and body mass at an air temperature of 0°C .

than differences in heat loss. In birds, thermoregulatory heat production predominantly involves shivering thermogenesis in the skeletal muscles (Dawson and Whittow, 2000). Although avian nonshivering thermogenesis has recently been reported, and may be involved in rewarming in hummingbirds, brown adipose tissue is not thought to be present in birds (reviewed by Bicudo et al., 2002). Hence, the differences in rewarming rates may reflect physiological differences in the efficiency of thermogenesis or differences in the scaling of the sources of thermogenesis.

Energetic Cost of Rewarming

The energetic cost of rewarming from torpor can be estimated using a simple biophysical model (Fig. 2). The model can be used to generate generalized predictions of E_{rewarm} using allometrically predicted BMR, thermal conductance, and rewarming rate but can also accurately predict E_{rewarm} in particular species by incorporating species-specific deviations from allometrically expected values (Fig. 2).

Our approach to modeling E_{rewarm} is more biologically realistic than previous models (Prothero and Jürgens, 1986; Humphries et al., 2002). These models did not account for heat loss during the rewarming process and can seriously underestimate E_{rewarm} (Fig. 2). Moreover, they make the unrealistic prediction that E_{rewarm} is constant at T_a s below the setpoint T_{torpor} .

Our model predicts that E_{rewarm} increases linearly with body mass for any T_{torpor} . However, when E_{rewarm} is expressed as a fraction of the predicted energy expenditure for an entire torpor bout (E_{torpor} ; A. E. McKechnie and B. O. Wolf, unpubl. data), $E_{\text{rewarm}}/E_{\text{torpor}}$ increases with body mass following a three-parameter power function, confirming that rewarming from torpor is much more energetically costly, in relative terms, for larger birds. For instance, during a 12-hour torpor bout at $T_a = 0^\circ \text{C}$ and with $T_{\text{torpor}} = 18^\circ \text{C}$, E_{rewarm} represents 11% of E_{torpor} in a 5 g bird, but 73% of E_{torpor} in a 500 g bird.

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Insect Cold-Hardiness: New Advances Using Gene Screening Technology

KENNETH B. STOREY AND DAVID C. McMULLEN

Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada

Abstract. The goldenrod gall moth *Epiblema scudderiana* uses the freeze avoidance strategy of winter cold hardiness. In recent studies we have begun to explore the changes in gene expression that support subzero survival, using both cDNA library screening and cDNA array screening technologies. Screening of a library prepared from cold-exposed larvae showed the cold-responsive up-regulation of EsMlp, a LIM protein with a probable role in myogenesis. Heterologous screening using *Drosophila* cDNA arrays proved highly effective in identifying multiple genes that responded to -20°C exposure, in particular highlighting the up-regulation of six plasma membrane transporters. Heterologous screening is an excellent search tool for seeking new genes/proteins that support stress tolerance in comparative animal systems, provided that appropriate validation and follow-up techniques are used.

Introduction

Many insects use the freeze avoidance strategy of winter survival and achieve deep supercooling by the production of antifreeze proteins and the accumulation of high concentrations of carbohydrate protectants such as glycerol. Research in my lab uses the goldenrod gall moth, *Epiblema scudderiana* (Lepidoptera, Olethreutidae), as a model for studies of the metabolic adaptations that underlie insect freeze avoidance, often comparing and contrasting these with the responses of the freeze tolerant goldenrod gall fly, *Eurosta solidaginis* (Diptera, Tephritidae) (Storey and Storey, 1992). Both overwinter as final stage larvae in galls on the woody stems of goldenrod and, as such, they are often exposed above the snow-

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line to the full force of ambient winter temperatures, which can fall to at least -30°C in the Ottawa area. Recent studies in my lab have been exploring the role of gene expression in phenomena, including freeze tolerance, anaerobiosis and hibernation (for review see Storey, 2003, 2004, this volume; Larade and Storey, 2002). Our recent work with both insect species has focused on the role of gene expression in adaptation to cold and/or freezing, and the present article presents highlights from some new studies of cold-induced gene expression in *E. scudderiana* derived from cDNA library and cDNA array screening.

Cold-induced Gene Expression: cDNA Library Screening

Changes in gene expression that support winter cold hardiness in insects could be triggered and regulated in one of two ways: (1) as seasonal responses that are initiated by photoperiod and/or thermoperiod cues and mediated by hormone signals, oftentimes as part of an obligatory winter diapause (particularly in univoltine species), or (2) as direct responses to low temperature exposure. The production of antifreeze proteins is a well-known example of the first mechanism as is the accumulation of the enzymatic machinery needed for cryoprotectant biosynthesis so that glycerol can be quickly synthesized when stimulated by cold exposures below 5°C . Indeed, a high proportion of gene expression responses that support insect cold hardiness are likely in place well before the first exposures to subzero temperatures occur. Our initial analysis of gene responses to acute cold exposure in both *E. scudderiana* and *E. solidaginis* actually supports this because we found relatively few genes that were putatively up-regulated from either cDNA library screening for cold-responsive genes in *E. scudderiana* or differential display PCR analysis of freeze-responsive genes in *E. solidaginis* (Bilgen, 1998; Bilgen et al., 2001).

Our first studies of cold-induced gene expression in *E. scudderiana* involved construction and screening of a cDNA library made from autumn-collected, cold-acclimated *E. scudderiana* using radiolabeled cDNA probes made from 15°C -acclimated (control) larvae versus larvae acclimated to 4°C for two weeks (cold-exposed) (Bilgen et al., 2001). Only three candidate clones were isolated as putatively up-regulated and only one of these encoded a full length protein that could be identified. This clone was shown to be the *E. scudderiana* homologue of the *Drosophila* gene *Mlp60A* and was named *EsMlp* (Genbank accession number AF206698). The translated amino acid sequence of *EsMlp* protein contained 94 amino acids and was 79% identical with the *Drosophila* protein. Like *Mlp60A*, *EsMlp* contained a single copy of the characteristic cysteine-rich

consensus sequence of LIM proteins that forms a pair of zinc fingers, as well as a glycine-rich region that further assigned it to the CRP subgroup of LIM proteins. Northern blotting revealed that *EsMlp* mRNA transcripts were elevated within eight hours when larvae were transferred from 4° C to -20° C, reaching 2-fold higher within 24 hours. Transcript levels also rose by approximately 3-fold over the autumn/winter to peak in February before falling to about 70% of autumn values in April. Western blotting revealed that EsMlp protein levels also increased by 3-to-4-fold in midwinter as compared with autumn levels but surprisingly, EsMlp protein in the larvae was 8.5-fold higher in April than in November. LIM proteins are involved in myogenesis during both embryogenesis and metamorphosis and play roles in the development and maintenance of cyto-architecture. *E. scudderiana* larvae are in diapause over the early winter months, so it is unlikely that metamorphic changes in muscle structure are occurring, but *EsMlp* up-regulation might have one of two functions over the winter: (1) a role in temperature-dependent restructuring of muscle protein composition, as typically occurs in poikilotherm acclimation to changing temperatures; or (2) a role in diapause development in the preparatory phases preceding the extensive myogenesis that will occur in the spring. Peak levels of EsMlp protein in April and the fact that the protein has a nuclear targeting signal and a zinc finger motif (often found in transcription factors) suggest that it may be involved in regulating the transcriptional program leading up to spring metamorphosis.

cDNA Array Screening for Cold-responsive Genes

The use of cDNA arrays for gene screening is currently one of the hottest techniques in biology and offers multiple benefits, including (1) simultaneous assessment of the responses by hundreds of genes, most of them identified, (2) detection of transcripts that are present in low copy number (library screening favors abundant transcripts), (3) relative ease of sample preparation and data quantification, and (4) the ability to assess both individual gene responses and cumulative responses by groups of genes (e.g., families, pathways, or cascades) (Eddy and Storey, 2002). This latter capacity is particularly important in studies of biochemical adaptation where we are seeking insights into the types of metabolic responses that underlie organismal responses to diverse environmental stresses. Recently, we have applied heterologous probing (array produced from one species—human, rat, *Drosophila*—but screened with cDNA from another) to analyze stress-induced gene expression in several systems. The technique has been criticized for several reasons: (1) cross-hybridization with spots on the array

is always less than 100%, (2) due to differential hybridization capacities comparisons of responses between genes are compromised, and (3) a small possibility of false positive matches exists (i.e., binding to cDNA that is not its homologue). However, the reality is that species-specific arrays are never going to be available for all organisms and that heterologous probing, if correctly validated, is a superb search tool. A substantial percentage of genes are highly conserved across phylogeny and even at considerable phylogenetic distances, strong cross-hybridization can occur. For example, after optimizing hybridization and washing conditions, we achieved cross-hybridization with human 19K cDNA arrays (Ontario Cancer Institute) that was 85–90% for cDNA from hibernating mammals, 60–80% for freeze-tolerant frogs, and 18% for the marine snail *Littorina littorea* (a freeze- and anoxia-tolerant species) (Storey, 2003; Eddy and Storey, 2002; Larade and Storey, 2002). For snails this percentage seems low, and in addition, only 10.6% of the genes that hybridized were designated as putatively up-regulated. However, with 19,000 genes on the array, the result still provided over 300 “hits” of putatively anoxia-responsive genes for future study.

In new studies we used *Drosophila* microarrays produced by the Canadian *Drosophila* Microarray Centre at the University of Toronto (containing cDNAs for 7,222 genes, www.flyarrays.com) to screen for gene expression responses to cold exposure in *E. scudderiana*. Clearly, this involved heterologous probing and is subject to the above concerns. However, after optimization of hybridization and washing conditions, we achieved 89% cross-hybridization between *E. scudderiana* cDNA and the *Drosophila* chip, which allowed us to evaluate the expression of over 6,400 genes on the array. The data discussed below assesses the effects of acute subzero temperature exposure on gene expression; controls were September-collected *E. scudderiana* larvae that were acclimated to 15° C for two weeks and experimental animals were larvae that were then acutely moved from 15 to –20° C and held for 24 hours. Total RNA was isolated from each group and first strand cDNA synthesis was performed in the presence of cyanine 3 or cyanine 5 fluorescent dyes. Labeled cDNA from control and experimental conditions was hybridized with the cDNA array for 16 hours at 30° C. After washing, fluorescence readings at two wavelengths were taken using a Vertek scanner, and Arraypro software was used to quantify fluorescence intensity and calculate the ratio of cy3:cy5 binding by each spot. Analysis of the data showed that 4.7% of cross-reacting genes were putatively up-regulated in the experimental condition by 1.5-fold or greater, whereas a similar number were down-regulated; hence, about 90% of transcripts are unaltered by cold exposure.

All data derived from heterologous probing require verification by other methods to confirm up-regulation, and ongoing studies in our lab are focused on selected genes or gene families that were highlighted by the array screening. A general methodology for verification is proving highly effective. For any new gene, this begins with a virtual trip to Genbank to retrieve sequences from several other species. From these, a conserved consensus sequence is derived and used to design a cDNA probe (commercially synthesized) that is used with the PCR reaction to retrieve and amplify the species-specific PCR product. After sequencing to confirm its identity, the species-specific product is used as the probe to measure relative mRNA levels under multiple conditions via quantitative PCR (or Northern blotting). With the further use of peptide antibodies designed and produced from the translated species-specific amino acid sequence, we can also analyze accompanying stress-induced changes in protein levels.

Genes that were highlighted from array screening as being strongly up-regulated by -20°C exposure of *E. scudderiana* larvae included a rather large number of plasma membrane transporters, among them:

1. **Concentrative nucleoside Na^+ transporter 2 (CNT2):** CNTs cotransport sodium and nucleosides unidirectionally against the nucleoside concentration gradient (Cabrita et al., 2002) and function in both salvage pathways for nucleic acid synthesis and in the transport of nucleosides that have important roles in signaling (e.g., adenosine plays a key role in metabolic rate depression; Storey, 2004, this volume).
2. ***Na* (*K*) *Cl* cotransporter:** These electroneutral cotransporters are nearly ubiquitous and have an important role in sodium chloride absorption/secretion and in cell volume regulation during hypertonic and other volume challenges (Kaplan et al., 1996).
3. ***Na*, *Pi* cotransporter:** Inorganic phosphate has critical roles, both in intermediary metabolism and in the structure of macromolecules. However, due to the negative electrochemical potential across the cell membrane, inorganic phosphate cannot enter cells by diffusion and hence is imported by Na^+ -dependent *Pi* cotransporters that have important roles in regulating phosphate availability (Werner and Kinne, 2001).
4. ***Na*-*Ca* exchanger (NCX):** These exchangers are found in the plasma membrane of most cells and are integral to Ca^{2+} homeostasis (Omelchenko et al., 2003). In heart, for example, NCX provides the principal mechanism for Ca^{2+} extrusion after contraction. NCX may also function in Ca^{2+} influx, particularly under pathophysiological conditions. Isoform 1 is found in a

macromolecular complex with protein kinase A, its anchoring protein AKAP, protein kinase C, and protein phosphatases 1 and 2A. Beta-adrenergic mediated phosphorylation of NCX increases exchanger current.

5. *Dicarboxylate transporter (cation-independent)*: This sodium-independent transporter in *Drosophila* was named INDY (I'm not dead yet) because mutation of the gene to create a disfunctional protein resulted in a two-fold extension of the average adult lifespan (Inoue et al., 2002). INDY transports a variety of tricarboxylic acid cycle intermediates, including citrate and succinate, and it is hypothesized that dysfunction of INDY reduces substrate availability for energy metabolism, resulting in life extension effects similar to those achieved by caloric restriction.

6. *Monocarboxylate transporter (MCT)*: Monocarboxylic acids such as lactate and pyruvate play central roles in metabolism and are often transported between tissues that produce versus catabolize these compounds. Their transport across the plasma membrane is via proton-linked MCTs that occur in especially high levels in white muscles and are also up-regulated by increased work load in heart (Halestrap and Price, 1999).

Coordinated up-regulation of a variety of plasma membrane transporters in response to acute subzero exposure is an intriguing response that has not previously been reported as a part of cold acclimation. Up-regulation may be needed to enhance the overall levels of transporters in the cold or perhaps to alter the relative levels of different isoforms of each transporter. Further studies will verify and explore changes in both mRNA and protein levels of each of these transporters and aim to discover how they contribute to reestablishing homeostasis at low temperature.

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Advantages and Disadvantages of Freeze-Tolerance and Freeze-Avoidance Overwintering Strategies

KARL ERIK ZACHARIASSEN, SINDRE ANDRE PEDERSEN, AND ERLEND KRISTIANSEN
Laboratory for Ecophysiology and Toxicology, Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway.

Abstract. The strategy of freeze-avoidance among insects seems to be promoted by a small body size and the ability to be inactive during cold exposure. Tolerance to freezing seems to be promoted by a large body size, exposure to extreme cold, and the combination of activity and cold exposure. Since frozen insects do not lose water to ambient ice, insects with a high cuticular water permeability should develop tolerance to freezing. There are also indications that insects with a high extracellular sodium concentration and insects that are exposed to high levels of trace metals might tend to develop the strategy of freezing tolerance.

Introduction

Ectothermic animals exposed to cold may use two strategies for survival. They may avoid freezing by supercooling or develop tolerance to freezing. In order to become cold-hardy, freeze-avoiding insects undergo a number of physiological changes that strongly enhance their capacity for supercooling. Although many species of insects avoid freezing by avoiding exposure to cold, the term freeze-avoiding should be reserved for those that are physiologically adapted to avoid freezing when exposed to low subzero temperatures. The term freeze-tolerant should be used for any insect, that tolerates staying frozen at or below the temperature where freezing is initiated.

Freeze-avoiding and freeze-tolerant organisms may be found in the same habitats, but it is not clear why some organisms seek to avoid freezing whereas

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others have developed freezing tolerance. In the present article the physiological and ambient factors that may promote one strategy or the other are discussed.

Summer organisms usually freeze at temperatures from -8 to -12°C (Zachariassen, 1985). These high supercooling points (SCPs) are caused by ice nucleating components (INAs) in cells, intestine, or in some cases the haemolymph. Freezing in these compartments creates an osmotic water influx, which causes swelling and eventually rupture of the compartment walls. Another injurious effect is the development of toxic solute concentrations (Zachariassen, 1985).

Freeze avoidance based on high supercooling capacity is found mainly among insects. To obtain the required high supercooling capacity, insects remove INAs from their intestine and cells (Zachariassen, 1985). As shown in Fig. 1, this brings their SCPs down to about -20°C . A further depression of the SCPs is

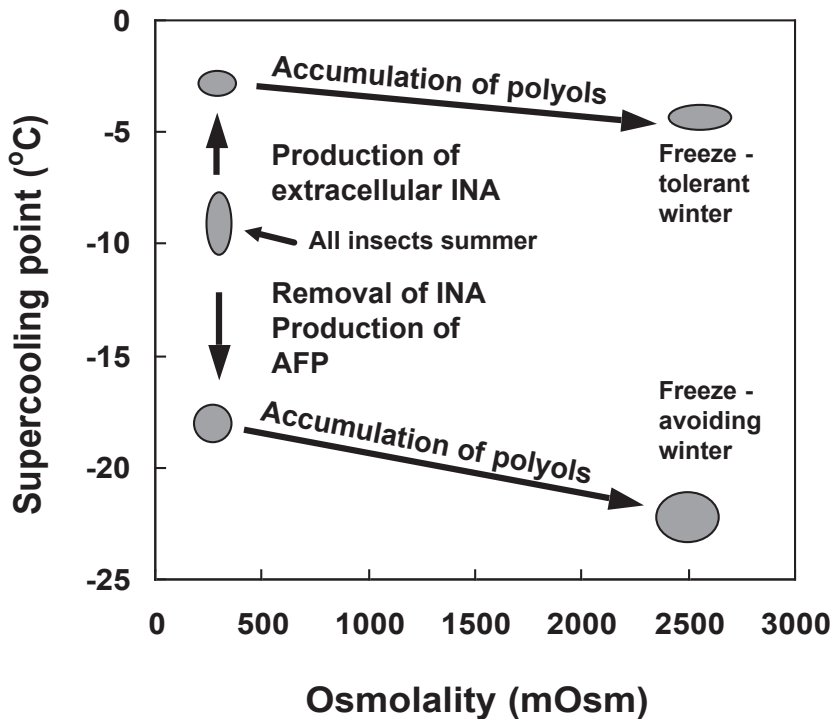


Fig. 1. Supercooling points of freeze-tolerant and freeze-avoiding insects as a function of their body fluid osmolality (from Zachariassen, 1980). INA: Ice nucleating agents. AFP: Antifreeze proteins.

obtained by the accumulation of polyols in their body fluids. The polyols cause a SCP depression that is about twice the corresponding depression of the melting point (MP), and insects with multimolal polyol concentrations in their body fluids may supercool to well below -30°C (Zachariassen, 1985; Gehrken et al., 1991). The supercooled state is stabilized by antifreeze proteins (AFPs), which are present in the hemolymph, intestine, and intracellular fluid (Duman, 2001; Kristiansen et al., 1999). The AFPs also prevent inoculation of external ice through the body wall (Gehrken, 1992; Olsen et al., 1988).

Many insect species are tolerant to freezing. Freeze-tolerant organisms prevent an injurious intracellular freezing by establishing a protective extracellular freezing at a high subzero temperature. The extracellular freezing is established by INAs that are present in the hemolymph (Zachariassen, 1985) or associated with the intestinal wall (Shimada, 1989), or by inoculation of external ice through the body wall (Gehrken et al., 1991).

Extracellular freezing causes an increase in extracellular solute concentrations, which in turn causes osmotic water efflux from cells and intestine. This brings intracellular and intestinal fluid into vapour pressure equilibrium with ice, thus preventing ice nucleation in these compartments (Zachariassen, 1985). However, when organisms are sufficiently cooled, concentrations of perturbing solutes, such as inorganic ions, may reach toxic levels. In many freeze-tolerant species, the concentrations of perturbing solutes are kept low by the accumulation of polyols, which reduce the amount of ice and thus salt concentrations coligatively (Zachariassen, 1979).

Effects of Body Size

Bigg (1953) showed that there is a linear relationship between the SCP and the logarithm of the volume of water samples (Fig. 2). As the volume increases by a factor of 10, the SCP increases by about 2.3°C . Hence a small body size presumably favours the strategy of freeze-avoidance. The data in Fig. 2 suggest that this is the case. Although there is a large range of overlap between the body masses of freeze-avoiding and freeze-tolerant insects, the largest insects seem invariably to be freeze-tolerant, whereas the smallest ones are invariably freeze-avoiding.

Fig. 2 also shows that the SCP of freeze-avoiding insects drops with diminishing body mass. Since it seems unlikely that large freeze-avoiding insects have more potent nucleating structures than small ones, the correlation may suggest that nucleation in freeze-avoiding insects is caused by water itself.

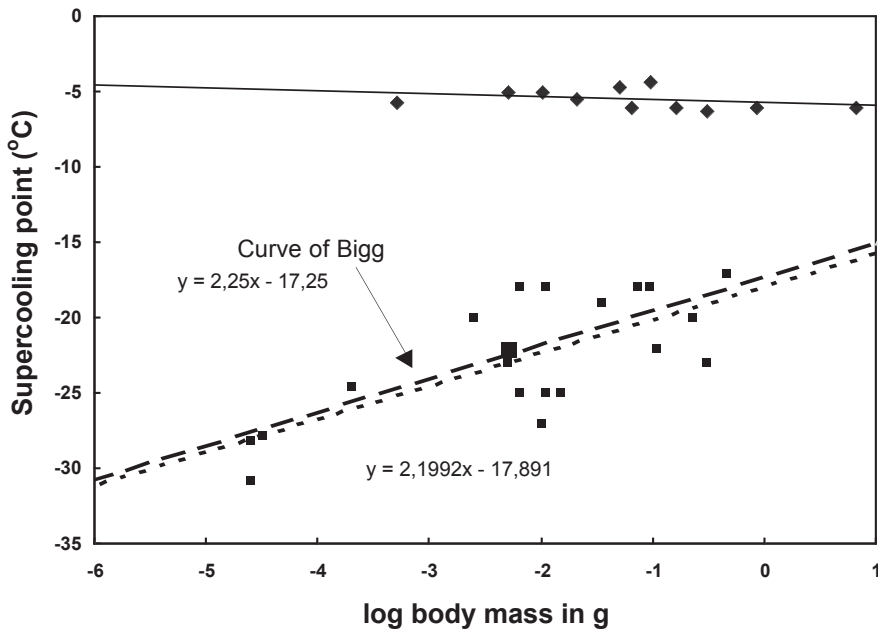


Fig. 2. Supercooling points of freeze-tolerant (◆) and freeze-avoiding (■) insects as a function of their body mass. The solid line is the linear regression line of freeze-tolerant species, whereas the widely broken line is the regression line of freeze-avoiding species. The narrowly broken line is the curve of Bigg, which represents supercooling points of highly rinsed samples of water (from Bigg, 1953).

Effect of Degree of Cold Exposure

The data in Fig. 3 show that the accumulation of polyols has different cold-hardening effects in freeze-tolerant and freeze-avoiding insects. At higher polyol concentrations, freeze-tolerant species become far more cold-hardy than freeze-avoiding ones. This should favour the strategy of freezing tolerance among insects living in extremely cold areas, a contention which is supported by the fact that cold continental areas like the inland areas of Canada and Alaska have relatively many freeze-tolerant species (Miller, 1982; Ring, 1982).

Combination of Cold Exposure and Activity

In alpine tropical areas there are daily temperature variations from -20 to $+20^{\circ}\text{C}$ throughout the year (Sømme and Zachariassen, 1981). Insects in these areas are active during day but experience subfreezing temperatures at night.

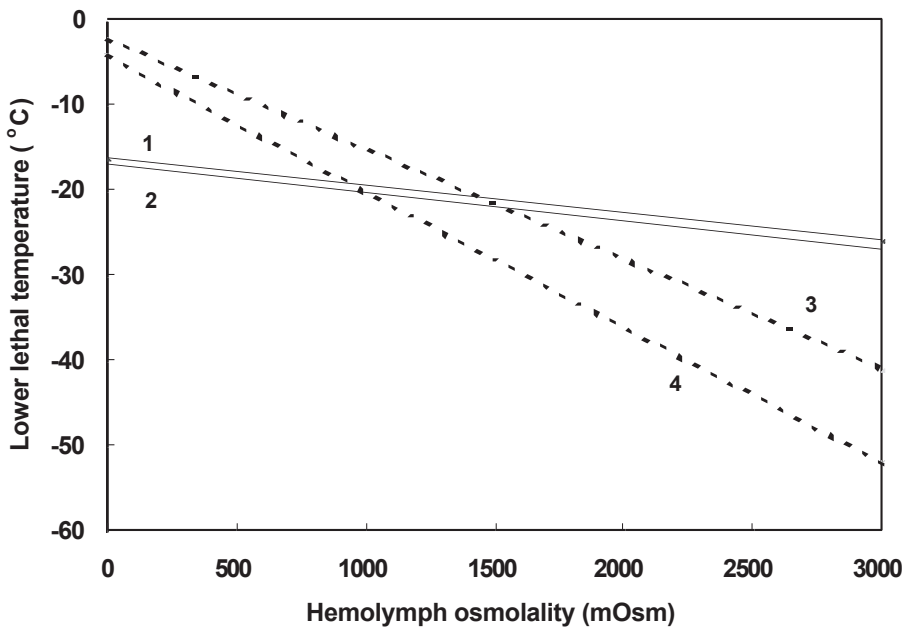


Fig. 3. Lower lethal temperatures of freeze-avoiding (solid lines) and freeze-tolerant beetles (broken lines), plotted as a function of body fluid osmolality. Line 1: 9 species of freeze-avoiding beetles (Zachariassen 1985). Line 2: Freeze-avoiding *Ips acuminatus*. Line 3: *Pytho depressus*. Line 4: *Phyllodecta laticollis*. The lower lethal temperature of freeze-avoiding beetles is identical to their supercooling point (from Zachariassen, 1985).

The daytime activity requires an intact metabolic system, and the insects cannot remove enzymes or other potential ice nucleating proteins from their systems. Furthermore, daily food intake leaves the intestine with ice-nucleating food particles during night. These insects cannot develop a high supercooling capacity, and their cold-hardiness strategy must be tolerance to freezing. This is probably the evolutionary drive behind the development of freeze-tolerance among cold exposed insects in tropical mountains (Sømme and Zachariassen, 1981; Duman and Montgomery, 1991). Alpine African species have been shown to have potent extracellular INAs, but the required intact metabolic system prevents them from becoming highly cold-hardy by accumulation of polyols (Storey and Storey, 1989). Since the organisms may spend the nights at protected sites, their moderate degree of cold hardiness is sufficient (Sømme and Zachariassen, 1981). Due to the access to protected habitat, many tropical alpine insects do

not display any particular adaptations to cold (Sømme and Zachariassen, 1981; Duman and Montgomery, 1991).

Alpine insects in the temperate region live under similar conditions in the summer, and several of these species are freeze tolerant even in the summer (Sømme and Conradi-Larsen, 1979; van der Laak, 1982).

Another group of insects that combine cold exposure and activity are the so-called winter active insects. In contrast to most other cold exposed animals they are active while exposed to cold, and they may be active on the top snow at temperatures down to -10°C . Since they do not accumulate polyols, the melting point of their body fluid is about -1°C , implying that they are active in a supercooled state (Hågvar, 1971, 1973). In this condition they eat and copulate, and winter active spiders may even make webs and catch winter active collembolans (Hågvar, 1973; Østbye and Sømme, 1972).

One should expect that these insects would depress their supercooling points well below the temperature range where they are active. However, the association between activity and ice-nucleating structures is reflected in a moderate capacity for supercooling. Their supercooling points are around -10°C , which is the range where active summer insects freeze. The fact that these insects are supercooled and in intimate contact with external ice suggests that the winter active insects are indeed living at the edge of disaster. This extreme situation seems to be met by the presence in their body fluids of antifreeze proteins, which prevent inoculation of external ice through the body wall (Husby and Zachariassen, 1980)

Effect of Water Balance

Lundheim and Zachariassen (1992) found that supercooled insects surrounded by ice lost significant amounts of water whereas frozen insects did not have any water loss. This is due to the fact that the vapour pressure in equilibrium with ice is lower than that in equilibrium with supercooled water at the same temperature. Many supercooled insects spend the winter in ice-laden hibernaculae, and if they are supercooled, body water will evaporate and join the external ice. In frozen insects the vapour pressure of the unfrozen fraction of the body fluids is in equilibrium with ice, and there will be no net exchange of water between the body fluids and the external ice. Lundheim and Zachariassen (1992) also found that the freeze-avoiding insects have adapted to this situation by having a low water permeability through their cuticle, whereas the freeze-tolerant insects have a considerably more leaky cuticle. Freezing tolerance is obviously favourable for insects with a high cuticular water permeability.

Effect of Extracellular Ionic Composition

All animals except some insects have Na as the dominating extracellular cation. Predatory insects, which feed on Na-rich animals, seem to have a high Na concentration, whereas insects feeding on low Na plants may have K or even Mg as the dominating extracellular cations (Jeuniaux, 1971; Sutcliffe, 1963). Among the low Na species there are both freeze-tolerant and freeze-avoiding species, but the high Na species seem with few exceptions to be tolerant to freezing (Baust and Miller, 1970; Ohyama and Asahina, 1972; Storey and Storey, 1989; Tursman et al., 1994; Whitmore et al., 1985).

One may speculate about the reasons why the species with a high extracellular Na concentration should prefer the strategy of freezing tolerance. Freeze-avoiding species with a low extracellular Na concentration seem to maintain their Na gradients even when they are cooled to low temperatures (Dissanayake and Zachariassen, 1980), and the presence of AFPs or high levels of other extracellular cations such as Mg may be the basis for this capacity (Zachariassen et al., 2004). These mechanisms may be unable to maintain the gradient when the extracellular Na concentration is high. A high extracellular Na concentration probably reflects a high transmembrane Na gradient, which is important for cellular extrusion of Ca and cellular accumulation of free amino acids. The Na gradient is maintained by the Na/K pump, and the large ATP consumption of this pump requires an intact metabolic system, which excludes the removal of enzymes with a nucleating capacity. Freezing tolerance may then be the most feasible cold-hardiness strategy.

Effect of Trace Metal Exposure

Organisms exposed to potentially toxic trace metals protect themselves by producing metal binding proteins such as metallothioneins (MTs), which reduce the levels of free metals in the body fluids (Cherian and Chan, 1993). MTs have a remarkably high content of the amino acid cysteine. Free cysteine usually occurs in relatively low concentrations in insects, and MT production may cause a strong depletion of the cysteine pool. Also the antifreeze proteins (AFPs) of many freeze-avoiding insects have a high content of cysteine (Duman, 2001). In metal exposed insects, MTs may have consumed so much cysteine that there is too little left to produce AFPs. Hence, metal exposure may favour freezing tolerance.

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Live and Let Diapause: Cell Cycle Regulation During Insect Overwintering

SAVVAS C. PAVLIDES, KENNETH A. WEIR, AND STEVEN P. TAMMARIELLO
Binghamton University (SUNY), Department of Biological Sciences, Binghamton, NY 13902

Abstract. The flesh fly, *Sarcophaga crassipalpis*, overwinters in a pupal diapause that is entrained by exposure to short photoperiod coupled with low temperatures. This stage is characterized by decreased metabolism and oxygen consumption along with a halt in morphogenesis. During this period of dormancy, brain cells in the flesh fly are arrested in the G_0/G_1 stage of the cell cycle; however, cell proliferation is reestablished within 12 hours after diapause termination. We report here the differential expression of two cell cycle regulatory genes, *pcna* and *cyclin e*, before, during, and after diapause in the flesh fly. These genes encode for proteins that control the G_1/S phase progression in eukaryotic cells and are highly expressed before and after, but not during, flesh fly diapause.

Insect Diapause

Most insect species living in temperate climates have evolved the ability to overwinter in a period of developmental stasis called diapause. This period is normally induced by exposure to short photoperiod coupled with low temperatures well in advance of diapause initiation (reviewed in Denlinger, 2002). While diapause is a period of metabolic shutdown and developmental stasis, a wealth of evidence has been published that defines diapause as an alternate developmental pathway and not simply a shut down in gene expression and protein production (Flannagan et al., 1998; Morita et al., 2003; Zhao et al., 2004). While the physiology of insect dormancy has been well established, studies defining the molecular control of this period of developmental stasis are in their infancy. The majority of diapause-associated genes isolated to this point encode for proteins that will be used by the

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insect to overcome unfavorable conditions during this long period of inactivity. Examples include the differential regulation of several heat shock proteins during diapause (reviewed by Denlinger, 2002) and reports of high production of specific storage proteins in insect hemolymph prior to diapause entrance (reviewed by Schoofs et al., 1997; de Kort, 1996). Our interest lies in the molecular regulation of development during this period. As a starting point, we have examined cell cycle control during the pupal diapause of the flesh fly, *S. crassipalpis*.

Cell Cycle Control in Eukaryotic Cells

Cell proliferation in higher eukaryotes is an ordered process controlled by a series of timed events involving intracellular signaling, gene expression, protein/protein interactions and proteolysis (reviewed by Murray, 2004). Cell proliferation is regulated by a specific set of proteins, called cyclins, along with their protein kinase counterparts called cyclin-dependent kinases (cdks). The cdk partner acts as a functional kinase while the cyclin recognizes the substrate to be phosphorylated (reviewed by Sherr, 2000). The G_1 to S phase transition is a critical point during the cell cycle, and entry into S phase is driven by the three classes of G_1 cyclin, cyclins D, E, and A (reviewed by Ekholm and Reed, 2000). While both cyclin D and cyclin E are necessary for S-phase entry in many eukaryotic cells, it appears that cyclin E is the more important of the two proteins during embryogenesis and development in the fruit fly, *Drosophila melanogaster* (Knobloch et al., 1994). Ectopic expression of cyclin E in *D. melanogaster* cells arrested in G_1 results in entry to the S phase, while ectopic expression of cyclin D in these cells fails to stimulate cells to progress into S phase (Richardson et al., 1995). The G_1 to S phase checkpoint is controlled by the negative regulation of the transcription factor E2F. This protein is bound and negatively regulated by the retinoblastoma (Rb) protein during the early G_1 phase. As the cell progresses toward the G_1 to S transition, cyclin D/Cdk4 and cyclin E/Cdk2 hyperphosphorylate Rb leading to the degradation of the Rb protein and the release of E2F (Fig. 1). At this point free E2F transcribes several S-specific genes including the proliferating cell nuclear antigen (PCNA) and DNA polymerase (Thacker et al., 2003). PCNA is a co-factor of DNA polymerase during the elongation stage of DNA replication, and it acts as a molecular "clamp" that interacts directly with both the DNA strand and DNA polymerase δ (Fukuda et al., 1995). The down-regulation of these genes results in the cell arresting in the G_1/G_0 stage, thus preventing entry into the S phase of the cell cycle (reviewed by Ekholm and Reed, 2000).

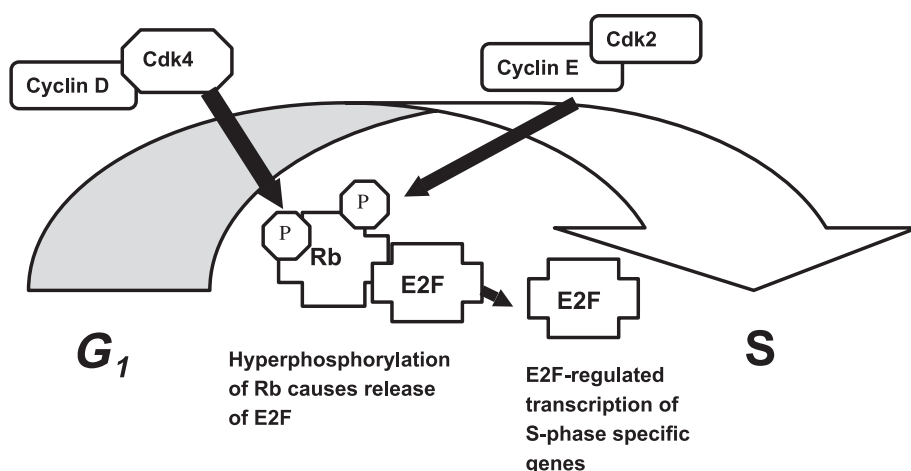


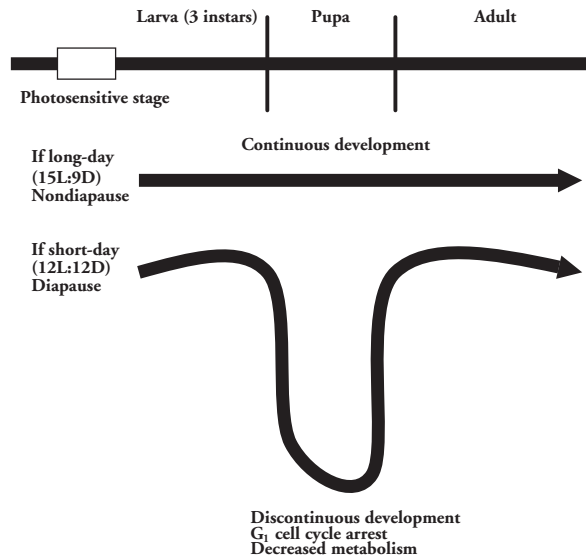
Fig. 1. The G_1 to S phase transition in eukaryotic cells is regulated by the hyperphosphorylation of Rb by cyclin D/Cdk4 and cyclin E/Cdk2. Release of E2F from Rb allows entrance into S phase.

Diapause-related Cell Cycle Arrest

The flesh fly overwinters in a pupal diapause that is entrained during a four-day photosensitive stage spanning the last two days of embryonic life and the first two days of larval life (Denlinger, 1985). Embryos exposed to short day-lengths (< 13.5 hours) during this photosensitive period will enter diapause as pupae (Fig. 2). Cell proliferation during pupal diapause in the flesh fly appears to be controlled at the G_1 to S phase boundary (Tammariello and Denlinger, 1998). Greater than 97% of cells in the brain are arrested in the G_1 phase of the cell cycle within 10 days after the onset of diapause, compared to approximately 75% in pre-diapause wandering larvae or in non-diapausing pupae that were reared under long day-length conditions. At the termination of diapause, cells begin to proliferate and adult development resumes.

In order to elucidate the mechanism underlying this cell cycle arrest, we performed semiquantitative RT-PCR to examine the expression patterns of *cyclin e* and *pcna* using mRNA samples extracted from non-diapausing pupae, as well as day five diapausers, day 50 diapausers and pharate adults that had terminated diapause. Both *pcna* and *cyclin e* are down-regulated throughout diapause, however the period of up-regulation between these two genes varies significantly. The level of *pcna* remains very low until diapause termination, while *cyclin e* appears to be up-regulated during late diapause (50 days after pupation) and is

Fig. 2. Diapause is programmed in the flesh fly by exposure to short day-length during the photosensitive stage during late embryonic and early larval life. Diapause-destined larval will develop almost normally until diapause initiation two days after pupariation. Adult development resumes following diapause termination.



continuously expressed until cell differentiation during the formation of adult structures (Fig. 3). These data are consistent with recent literature suggesting that Cyclin E and PCNA are rate limiting and essential for the G₁ to S phase progression in the fruit fly, *Drosophila melanogaster* (Richardson et al., 1995; Thacker et al., 2003). Diapause-associated cell cycle arrest has also been reported in the tobacco hornworm, *Manduca sexta* (Champlin and Truman, 1998) and the silkworm, *Bombyx mori* (Nakagaki et al., 1991), however both arrests are at the G₂/M transition of the cell cycle and therefore may be under the control of a different set of gene products.

Concluding Remarks

Here we show a correlation between the expression of G₁ to S phase regulatory genes, cell cycle progression and developmental stasis during diapause in the flesh fly. It is apparent that cell cycle regulation represents a mechanism that is highly conserved between models of invertebrate dormancy; however, the exact stage at which cells are arrested appears to be species-specific. We hypothesize that the down-regulation of *pcna* and *cyclin e* is responsible for the negative regulation of the G₁ to S phase transition during diapause in the flesh fly, *Sarcophaga crassipalpis*. But is this G₁ cell cycle arrest sufficient for the diapause-related developmental arrest, or is it simply a consequence of a different mechanism controlling development during diapause in the flesh fly?

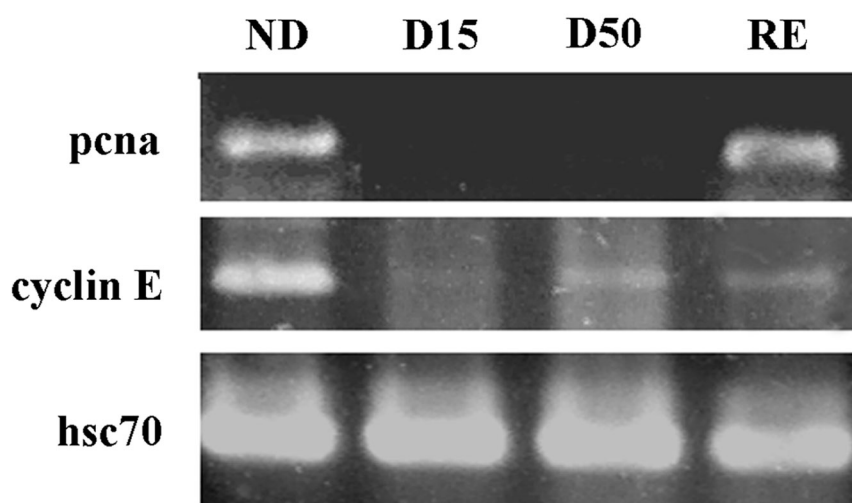


Fig. 3. Expression pattern of *pcna* and *cyclin e* in non-diapause (ND), day five diapause (d5), day 50 diapause (d50), and pharate adult (RE). Semi-quantitative RT-PCR was performed using experimental (*pcna*, *cyclin e*) primer sets along with a control primer set (*hsc70*). *Pcna* and *cyclin e* are down-regulated during diapause and expression remains low until the termination of diapause. Expression of the heat shock 70 cognate gene (*hsc70*) remains unaffected during diapause.

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Vertebrate Freeze Tolerance: Role of Freeze-Responsive Gene Expression

KENNETH B. STOREY

Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada

Abstract. Biochemical adaptation for freezing survival by wood frogs, *Rana sylvatica*, includes altered expression of a variety of genes whose protein products appear to address issues including cell volume regulation, ischemia resistance, cryoprotectant distribution, metabolic consequences of extreme hyperglycemia, and the potential for ice damage to tissues. Two techniques of gene discovery have been particularly useful: screening of cDNA libraries allowed discovery of three novel genes that support freeze tolerance whereas cDNA microarray screening is revealing new features of organ response to freezing and the involvement of multiple signal transduction pathways.

Introduction

Natural freeze tolerance is an important component of winter survival for several species of terrestrially hibernating frogs as well as selected turtles, lizards, and snakes (reviewed in Storey and Storey, 1996). Research in my lab focuses on the biochemical adaptations that support freezing survival using as our main model the wood frog, *Rana sylvatica* (Storey and Storey, 2004). Among others, our studies have explored cryoprotectant (glucose) metabolism, energetics and ischemia resistance, antioxidant defense, enzyme regulation, and signal transduction. At present, our major focus is the identification of genes (and their protein products) that are up-regulated to support freezing survival. Seasonal changes in gene expression are important preparatory events; for example, cryoprotectant synthesis and distribution is aided by a seasonal rise in glycogen phosphorylase activity in liver (to support rapid glucose synthesis) and elevated numbers of

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glucose transporters in the plasma membranes of all organs (to ensure rapid uptake of cryoprotectant). Other gene expression responses are triggered directly in response to freezing and address various consequences of extracellular freezing including cell volume reduction, ischemia, extreme hyperglycemia, and potential ice damage to tissues (Storey, 2004; Storey and Storey, 2001). Our first studies of gene expression used screening of a cDNA library made from liver of frozen frogs and identified the freeze-responsive up-regulation of the genes for fibrinogen alpha and gamma subunits, the mitochondrial ADP/ATP translocase and a novel gene called *fr10* (reviewed in Storey and Storey, 2001) (Table 1). Further screening of the liver library revealed a number of other freeze-responsive genes including the mitochondrial inorganic phosphate carrier (PiC) (DeCroos and Storey, in press), NADH-ubiquinone oxidoreductase subunit 4 (ND4) and elongation factor 1 gamma subunit (EF-1 γ) (S. Wu and K. Storey, unpublished data) as well as two more novel genes that we named *li16* and *fr47* (McNally et al., 2002, 2003). The identification of two mitochondrial transporters as up-regulated in liver, as well as others revealed from cDNA array screening (Storey, 2004), is beginning to suggest that mitochondria must have unique ways of dealing with the dehydration and ischemia stresses that accompany freezing. Notably, mitochondria are impermeable to glucose and so the extreme hyperglycemia (100–300 mM glucose in unfrozen cells, rising substantially when extracellular freezing occurs) in the cytoplasm that defends a minimum cell volume against the extracellular accumulation of ice would impose a huge osmotic stress on mitochondria. One of our current challenges is to determine how the freeze-responsive up-regulation of several mitochondrial membrane transporters fits into this picture.

Novel Genes

One of the critical advantages of cDNA library screening as a technique of gene discovery is its ability to target novel stress-responsive genes and, in the case of wood frogs, three such genes have been found and named *fr10*, *fr47* and *li16* (Cai and Storey, 1997; McNally et al., 2002, 2003). They have no similarity to any sequence in Genbank nor to each other, and all three have distinctly different profiles of gene and/or protein expression with respect to stress-responsiveness (freezing, anoxia, dehydration), organ-specificity, response to second messengers in tissue incubations, and structural characteristics (Storey, 2004). All are strongly up-regulated in liver during freezing, *li16* transcripts also rise in heart and gut, and *fr10* was found in all organs tested. Transcripts of *fr47*

Table 1. Genes identified as freeze-responsive in wood frog heart or liver from cDNA library screening or cDNA array screening using human 19K cDNA gene chips (Ontario Cancer Institute); up-regulation was assessed from a comparison of tissues from control (5° C acclimated) versus frozen (24 hours at -3° C) frogs.

Function	Name
<i>Heart:</i>	
Glucose-related	Facilitated glucose transporter type 4 (insulin responsive), Glucose-6-phosphatase
	Aldo-keto reductase, Receptor for advanced glycosylation end products (RAGE)
Ischemia/hypoxia related	Adenosine A1 receptor, Adenosine A2A receptor, 5' Nucleotidase
	Hypoxia-inducible factor 1 alpha subunit, ATP synthase Fo subunit c isoform 3
Fluid dynamics	Atrial natriuretic peptide receptor
Transmembrane carriers and ion motive ATPases	Mitochondrial adenine nucleotide translocator, Monocarboxylic acid transporter; Na ⁺ -K ⁺ ATPase alpha 3 subunit
Antioxidant defense	Ferritin light chain, Glutathione-S-transferase theta 1 isozyme
	Thioredoxin, Metallothionein 1G, Glucose-6-P dehydrogenase
<i>Liver:</i>	
Damage repair	Fibrinogen α and γ subunits
Mitochondrial proteins	Adenine nucleotide translocator, inorganic phosphate carrier
	NADH-ubiquinone oxidoreductase subunit 4
Protein synthesis	Elongation factor 1 gamma subunit
Intracellular signaling	Phosphoinositide 3-kinase (PI3K) class 2 alpha polypeptide
	Casein kinase 2, Protein phosphatase 5, Multiple inositol polyphosphate phosphatase
Novel proteins	FR10, FR47, Li16

are liver-specific but are also found in other freeze-tolerant frogs, *Hyla versicolor* and *Pseudacris crucifer* (but not freeze-intolerant species), which suggests that FR47 protein addresses a freeze-specific problem (McNally et al., 2003). This suggestion is supported by the fact that FR47 protein increases in wood frog liver during freezing but not in response to either of two component stresses of freezing, anoxia or dehydration, when these are applied separately. Transcripts of *fr47* responded *in vitro* to incubations that promoted protein kinase C activity. *Fr10* transcripts respond strongly to both freezing and dehydration stresses, which suggests that the protein may be involved in volume regulatory or membrane stabilization responses during freezing. By contrast, both *li16* transcripts and Li16 protein respond strongly to freezing and anoxia stresses, and transcripts are up-regulated during *in vitro* incubation of liver slices with cyclic GMP; these results suggest a function for the protein in some aspect of ischemia resistance during freezing. We are continuing to explore the possible roles of these three proteins in wood frog freezing survival. One interesting outcome of these studies is the implication of protein kinases C and G in freeze-responsive gene expression which, together with the documented role of protein kinase A in cryoprotectant biosynthesis (Storey and Storey, 2004), implicates multiple signal transduction pathways in the metabolic adjustments needed for freezing survival, a conclusion that is also supported by the data from cDNA array screening presented below.

Freeze-responsive Genes Identified by cDNA Array Screening

In recent work we have applied heterologous probing using human cDNA arrays (containing over 19,000 cDNAs for known genes or expressed sequence tags) produced by the Microarray Center of the Ontario Cancer Institute (www.uhnres.utoronto.ca/services/microarray/) to evaluate freeze-responsive gene expression in wood frog organs (Storey, 2004, and below). For a discussion of the procedures involved in array screening, the advantages and disadvantages of heterologous probing, and the verification of putatively up-regulated genes, see Storey and McMullen (this volume).

Heterologous screening with cDNA prepared from heart (Storey, 2004) or liver (discussed below) of control (5° C acclimated) versus 24 hours frozen (at -2.5° C) frogs indicated dozens of genes that are putatively up-regulated by 2-fold or more during freezing. Several of these were already known to us from cDNA library screening or direct assay of enzyme/protein activities (e.g., ATP/ADP translocase, glucose transporters, glucose-6-phosphatase) and this helped

to confirm that array screening is a valid method for identifying freeze responsive genes. Furthermore, any of the putatively up-regulated genes that we have followed up to date have subsequently been confirmed as up-regulated by other methods (quantitative PCR, northern blotting) using species-specific cDNA probes and then peptide antibodies produced from the species-specific translated amino acid sequence.

We are currently using cDNA array screening to assess patterns of freeze-responsive gene expression in selected organs of wood frogs. The results suggest that the adaptations needed for freezing survival extend into many different areas of cellular metabolism and are providing us with many leads for future research. For example, genes identified as freeze-responsive from array screening of wood frog heart (Storey, 2004) include those that address the following functions (Table 1):

1. **Ischemia/hypoxia defense:** 5' Nucleotidase, which synthesizes adenosine as well as adenosine A1 and A2A receptors were up-regulated. Adenosine, particularly acting via A1 receptors, has a role in metabolic rate depression; adenosine signaling is known to suppress the activity of ATP-dependent ion channels and increases myocardial tolerance of ischemia.
2. **Antioxidant defense:** Up-regulation of iron and metal binding proteins (ferritin, metallothionein) was indicated along with antioxidant agents (thioredoxin, glutathione-S-transferase).
3. **Fluid dynamics:** Up-regulation of the receptor for atrial natriuretic peptide (ANP) suggests a role for this peptide, that is synthesized and secreted by heart, in orchestrating physiological responses to changing fluid dynamics during freezing. ANP responds to volume expansion and pressure overload and is a potent vasodilator that also increases glomerular filtration rate. Notably, ANP works through a cGMP-mediated pathway (known to stimulate *li16* expression) and is also known to reduce ischemia/reperfusion damage.
4. **Cryoprotectant accumulation:** The insulin-responsive glucose transporter isoform 4 is up-regulated and would act to increase the capacity for cryoprotectant uptake into heart during freezing.
5. **Response to extreme hyperglycemia:** Up-regulation of the receptor for advanced glycation end products (RAGE) suggests that the nonenzymatic glycation of proteins occurs in wood frogs as a consequence of high levels of glucose. Glycation of proteins is a major cause of diabetic vasculopathy, cataract, and other damage (caused by glycation of long-lived proteins) in man and other vertebrates and this response by RAGE suggests that wood frogs

may have adaptations in place that address this issue. Interestingly, a secretory version of RAGE has recently been found that is able to capture advanced glycation end products and neutralize their actions on the endothelial cells of blood vessels (Sakurai et al., 2003). Although still speculative, this form of RAGE acting in wood frogs during freezing could be of high adaptive value for preventing glycation damage to proteins.

Array screening using cDNA from wood frog liver has similarly documented the putative up-regulation of a variety of proteins during freezing (Table 1). One of the outstanding features of the liver data is that a large number of components of signal transduction pathways were highlighted. This, together with the previous identification of PKA, PKC, and PKG involvement in liver response to freezing, suggests high metabolic activity by liver in making multiple proactive responses to freezing that both protect the liver itself and provide secreted products (glucose, proteins) that contribute to whole animal cryopreservation. Responses by several signal transduction genes are noteworthy:

1. **Phosphoinositide 3-kinase (PI3K) class 2, alpha polypeptide:** PI3Ks are important components of intracellular transduction; they function downstream of receptor tyrosine kinases to mediate stimulation from growth factors, cytokines, and chemokines. Class 1A PI3Ks, which produce phosphatidylinositol-3,4,5-trisphosphate (PIP₃), are well known as a key link in the insulin-mediated activation of protein kinase B that, in turn, mediates several cell responses to insulin (Cantley, 2002). Less is known about the role(s) of Class 2 PI3Ks and the signal transduction pathways that they affect, but selected studies have reported their response to epidermal growth factor and to cytokines including TNFalpha and leptin (Ktori et al., 2003). The role of class 2 PI3Ks in freeze tolerance remains to be determined.
2. **Casein kinase 2:** CK2 is a ubiquitous protein kinase with a huge number of physiological substrates, many of them transcription factors or other proteins associated with gene expression or protein synthesis. Recent evidence suggests that CK2 plays an important anti-apoptotic role in cells (Litchfield, 2003) and its up-regulation under the conditions of ischemia and dehydration that accompany freezing may be important in preventing apoptosis under these stressful conditions.
3. **Protein phosphatase 5:** Discovered only seven years ago, this phosphatase which is stimulated by arachidonic acid and inhibited by okadaic acid has been linked with steroid receptor signaling and with the regulation of plasma membrane ion channels and the ANP receptor (Chinkers, 2001). Its natural

substrates and physiological role are poorly defined to date but its association with the ANP receptor could indicate an role in cell volume regulation during freeze/thaw.

4. Multiple inositol polyphosphate phosphatase (MIPP): Inositol-1,4,5-trisphosphate (IP_3) is a potent regulator of Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} is, in turn, a key intracellular second messenger. IP_3 and its co-product diacylglycerol (DAG) are well known as products of phospholipase C action on membrane phosphatidylinositol 4,5-bisphosphates. DAG plus the Ca^{2+} released by IP_3 then activate protein kinase C at the plasma membrane surface. MIPP action provides an alternative way of producing IP_3 from "higher" inositol polyphosphates (e.g., inositol-1,2,3,4,5- P_5) and therefore an alternative mechanism for modulating IP_3 , and hence Ca^{2+} , concentrations in cells (Yu et al., 2003). IP_3 levels are strongly elevated by freezing and anoxia stresses in wood frog liver (Holden and Storey, 1996, 1997) and MIPP up-regulation suggests that IP_3 levels, and hence Ca^{2+} -dependent signal transduction, may be modulated in at least two ways during freezing.

Overall, these new data on freeze-responsive gene expression demonstrate that natural freezing survival is a complex phenomenon that involves multiple signal transduction pathways, adjustments to numerous cell functions, up-regulation of many different genes, and the expression of selected novel genes.

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Ice, Antifreeze Proteins, and Antifreeze Genes in Polar Fishes

ARTHUR L. DeVRIES

University of Illinois, Department of Animal Biology, Urbana-Champaign, Illinois

Abstract. The ice-covered polar marine waters are the coldest waters in the world. Fishes living in these waters are fortified with antifreeze proteins (APs), which are either glycoproteins (AFGPs) or small proteins (AFPs) that lower their freezing points below that of the seawater but have little effect on their melting points. As expected, fishes living in the most extreme environments have the highest blood concentrations of AP. Seasonal variation in levels of AP occurs in temperate-water fishes, whereas in the high latitudes AP levels remain high throughout the year. The APs function by adsorbing to ice crystals that occasionally enter the fish's circulation through lesions in the gill or intestinal tract and inhibit growth at specific ice crystal faces. Antifreeze-coated crystals are sequestered in the spleen where they may be slowly dissolved by some as-yet unidentified mechanism. The antifreezes are synthesized by specialized cells in the stomach and pancreas and secreted into the digestive fluid as well as into the circulation. The APs are encoded in large gene families. The AFGPs in the Antarctic notothenioid fishes appear to have originated some 15 million years ago by modification of part of a trypsin-like protease gene; however, origin of the identical AFGPs in the northern gadoid fishes has not been identified. The AFPs have evolved independently several times, some by duplication followed by modification of preexisting genes such as sialic acid synthetase, lectins, and apolipoprotein genes.

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Polar Freezing Environments

The high-latitude waters of the Antarctic Ocean, in contrast to the Arctic Ocean, are often adjacent thick (500 m) ice shelves that generate freezing waters at their underside and thus are at their freezing point throughout the year (Hunt et al., 2003). The sporadic outflow of ice shelf water that is advected towards the surface generates supercooled water that nucleates and produces an abundance of minute ice crystals at any depth shallower than the bottom of the shelf. Although the freezing point of the surface water is -1.9°C , at the underside of the shelf, it can be as low as -2.3°C because of the effect of pressure on the freezing point (Fujino et al., 1974). Although the high-latitude Arctic Ocean lacks ice shelves, most of its surface is ice covered even during the summer and the surface waters are also at their freezing point. Many polar fishes have adapted to live in these extreme ice-laden environments, and the key evolutionary innovation that lead to their survival and colonization of these environments was the evolution of an effective biological antifreeze system. This adaptation was first clearly documented in the fishes inhabiting McMurdo Sound, Antarctica, where ice cover and low temperatures are at their extremes (DeVries, 1971).

Antifreeze Proteins

Fishes synthesize either a glycoprotein (AFGP) or a protein antifreeze (AFP) that circulates in their blood and is present in many other body fluids (DeVries, 1982). The AFs lower only the freezing point or temperature of ice growth but have little effect on the melting point (DeVries, 1971). Those species living in the most extreme environments have the highest blood AP concentrations (35–40 mg/ml) and they remain high throughout the year since they are required for survival. Those living in more temperate waters have considerably less circulating AP (DeVries, 1982). Where extensive environmental seasonal temperature variation occurs, antifreeze levels increase with decreasing temperature during the winter and disappear during the summer (Petzel et al., 1980; Fletcher, 1981). In high-latitude species that are exposed to freezing seawater throughout the year, artificial warm acclimation has little effect on circulating levels of the antifreeze indicating synthesis is constitutive and that adaptation to the extreme constant, cold over evolutionary time has resulted in a loss of a response to thermal change (DeVries and Lin, 1977). Heat shock responses in the Antarctic notothenioids also appear to be muted and has likewise been lost during adaptation to this constant cold environment (Hofmann et al., 2000).

Antifreeze proteins are either AFGPs or AFPs. The structure of the AFGPs is highly conserved, and in the antarctic notothenioid and arctic true codfishes they are nearly identical in terms of residue sequence and numbers of each size that vary from 2,600 to 35,000 D (Chen et al., 1997a,b). Only in the Antarctic notothenioid fishes does a combination of the two basic types constitute the antifreeze system (Jin, unpublished). The AFP component of the notothenioid antifreeze system is a 15 KD heat labile protein and by itself exhibits rather low antifreeze activity but in conjunction with the large AFGPs it amplifies their activity by approximately two-fold. This amplification or potentiation occurs in the presence of only a small amount (2–4 mg/ml) of this protein that we have termed antifreeze-potentiating protein (AFPP). Although found in most all the Antarctic notothenioid fishes, it is present at the highest concentrations (4 mg/ml) in those fishes living in the iciest environments (DeVries, unpub.). In the warmer northern areas of the Southern Ocean where fish still retain some AFGPs, the concentration of the AFPPs is much less and thus its role unimportant. The AFPP/AFGP complex strongly affects the growth of ice crystals in that it promotes crystal size dependence; the smaller the seed ice crystal the greater the potentiation effect. In its presence antifreeze activity is also strongly dependent upon the rate at which a small seed ice crystal is cooled; the slower the cooling rate the greater the antifreeze activity. Small crystal size and cooling rate dependence are relevant to freeze avoidance because in nature the fishes' environment contains an abundance of very small ice crystals and in addition water temperature changes slowly because the ocean is such a large heat sink.

Interestingly, if pure antifreeze potentiating protein is added to the blood of the arctic gadoids that only synthesize AFGPs, then a substantial additional lowering of their blood freezing point occurs, indicating that the effect of the AFPP is specifically one of boosting the antifreeze activity of the AFGPs. In contrast to the antarctic notothenioids, all other polar fishes appear to make use of a single class of antifreeze protein although there may be several different size isoforms (DeVries, 1986) and the structures are a helical coil, a globular molecule or a flattened molecule (Fletcher et al., 2001)

Adsorption-Inhibition Mechanism of Antifreeze Activity

The most plausible mechanism of antifreeze activity is the one developed by Raymond and DeVries (1977) where it was shown that AFPs bind to ice crystals and inhibit their growth on the preferred growth faces of hexagonal ice. Where definitive AFP structures have been determined, there is good evidence for

specificity between the AFP molecule and a certain face of hexagonal ice. With the winter flounder *Pleuronectes americanus*, a lattice match has been identified between the polar threonine residues and the oxygens of ice on a pyramidal plane (Knight et al., 1991). In this completely helical molecule, regular threonine repeats are separated by 16.3Å, a spacing that also separates specific oxygens of the water molecules on a pyramidal plane (20 $\bar{2}$ 0) of hexagonal ice. If the threonine residues are removed or replaced by another residue then the antifreeze activity is diminished and in some cases completely abolished (Haymet et al., 1999). Similar lattice matches have also been proposed for other APs, although the lattice match relationship is not as straightforward as it is with the winter flounder helical peptide (Knight and DeVries, 1994; Knight et al., 1993).

Adsorption of APs to ice is thought to divide the surface of an ice crystal into many small highly curved domains. The water molecules in the highly curved domains have fewer neighbors to hydrogen bond to than those along a straight growth front and thus those in a curved domain have a tendency to escape into the liquid phase. In order for them to remain in the curved front the temperature must be lowered which in effect is lowering the local freezing point (Raymond and DeVries, 1977; DeVries, 1984; Wilson, 1993). Different APs have different potencies in terms of the strength of binding to ice and different ice-face binding specificities, and these can be correlated with antifreeze activity. Those that show weak adsorption allow growth in the hysteresis gap, resulting in hexagonal bi-pyramids, while those with strong adsorption permit little if any growth in the hysteresis gap (Raymond et al., 1989). Those that alter the ice growth surface so that they cover almost all possible growth sites are the most efficient antifreezes. In the case of the combination of the AFGPs and AFPPs in the Antarctic notothenioids, the surface of an ice crystal appears to be completely coated thus effectively eliminating almost all growth sites (unpublished A. DeVries). It is not unexpected that antarctic fishes living in the most extreme environment would have the most potent and effective AFs.

Fate of Endogenous Ice

The adsorption-inhibition model implies that ice must be present within the fish in order for the ice-inhibition mechanism to work. Fishes inhabiting the environmentally extreme McMurdo Sound, Antarctica, do indeed contain ice; however, most of it is located on the skin and in the intestinal tract (DeVries and Cheng, 1992), sites that are in intimate contact with the environmental ice. The intestinal tract is really a tube that is open to the environment, and its presence

there is readily explained by the fact that these fishes drink seawater, in order to regulate body water and ion content (DeVries, 1988). To prevent solidification of the intestinal fluid, these fish fortify it with AFGPs as the process of water and ion uptake in the gut leaves the fluid strongly hypoosmotic to seawater (O'Grady et al., 1982). The sources of the intestinal AFGPs are the anterior stomach and pancreatic cells, and the latter that export them to the anterior end of the small intestine via the pancreatic duct. Amazingly the AFGPs appear to be immune to digestion and thus are passed out with the feces as there appears to be no reabsorption (O'Grady et al., 1982). Thus there is an energetic cost associated with the protection of the intestinal fluid but nevertheless a necessary one. Although there is loss of APs via the feces, loss of the circulating APs is prevented by elimination of filtration in the process of urine formation. Many polar fishes' kidneys are aglomerular (Dobbs and DeVries, 1975), and those that are glomerular have become functionally aglomerular (Eastman et al., 1979) and therefore neither filters out blood-borne APs.

The fact that no ice is found in any of the internal tissues with the exception of the spleen would suggest that ice entry is infrequent. Ice entry is thought to occur at lesions in the skin, gill, or gut. The fact that it has never been found in the blood or muscle indicates that it must be removed from the tissues and transported via the blood to the spleen in a timely manner. It is possible that macrophages of the immune system may recognize antifreeze-coated ice crystals, endocytosis them, and transport them to the spleen. The number of ice crystals present in the spleen of specimens of *Pagothenia borchgrevinki*, a fish that inhabits the sub-ice platelet layer, has been determined. The spleen of fish taken from the ice-laden surface waters during the coldest time of the year contain only 10 to 100 crystals or nucleation sites per spleen (Hunt et al., unpublished).

Reintroduction of ice-free *P. borchgrevinki* into the freezing surface waters results in accumulation of splenic ice but only after several days exposure. However, within a matter of hours the skin and intestinal fluid test positive for ice. The results of such studies strongly suggest that the entry of ice is slow and infrequent, and that the integument and gill surfaces have a strong resistance to its entry (Turner et al., 1985). Furthermore, exposure to water masses actively forming minute ice crystals at temperatures substantially below the surface seawater freezing point -1.9°C may be the unique driving force for ice entry since exposure to large surface area ice crystals in aquaria at freezing temperatures fail to yield splenic ice. Consistent with the interpretation of these recent findings are the observations that only about half of the common benthic species liv-

ing in the shallow waters test positive for splenic ice. In their habitats, patches of anchor ice occur only in areas where the bottom topography forces the supercooled underlying water masses up near the surface, generating conditions leading to an abundance of ice crystals in the water column and the associated formation of patches of anchor ice platelets. Thus the habitat of benthic fishes in contrast to *P. borchgrevinki* only occasionally experience subfreezing water masses saturated with minute ice crystals. Deep-water fishes in the present-day environmental regime are not exposed to ice crystals at their habitat depth (500 m) and thus their spleens are ice-free. Although their relatively high levels of antifreeze would appear superfluous, they may still be needed for occasional encounters with ice during their life cycle such as the juvenile planktonic stages. An alternate hypothesis is that the AFs are holdovers from a past glacial maximum when the ice cover was more extensive and ice shelves were grounded at the edge of the continental shelf, resulting in an abundance of ice in their deep-water habitat.

Origin and Evolution of Antifreeze Proteins

There are five types of APs that have evolved, including the AFGPs and four AFPs (type I, II, III, and IV) (Fletcher et al., 2001). The APs are encoded in the various fish genomes as families of linked genes separated by variable length intervening sequences. Although it is difficult to accurately determine the number of genes in most cases, best estimates indicate that there are as many as 150 copies present in some cold-water fishes. The AFGPs have independently evolved in the arctic gadids and in the antarctic notothenioid fishes (Chen et al., 1997b). In the latter it evolved from a trypsinogen-like serine protease (TLP) and in the unrelated northern true cods it is yet to be determined. The notothenioid AFGP coding sequence (ala-ala-thr_n) arose by *de novo* duplications of a rudimentary Thr-Ala-Ala coding element located at the junction of intron one and exon two of the TLP gene. This element probably underwent repeated duplications via replication slippage to create the repetitive AFGP polypeptide (Chen et al., 1997a). The formation of the incipient AFGP gene involved recruitment of the front and the tail segments of the ancestral protease gene to provide the secretory signal and down stream sequence and the deletion of the bulk of the protease gene's exons and introns. Recently evolutionary intermediates in the form of a chimeric protease-AFGP gene have been isolated from the genome of these fishes, validating the proposed mechanism of AFGP evolution (Cheng and Chen, 1999).

In the smelt, herring, and sea raven, the type II AFPs appear to have arisen from a Ca^{+} dependent lectin ancestor (Ewart et al., 1998). In the case of the longhorn sculpin, its AFP is related to a serum apolipoprotein where several 22 mer repeats exist, each beginning with a characteristic proline residue (Cheng and DeVries, 2002). The globular type III AFP of the eelpouts has sequence identity with sialic acid synthetase (Baardsnes and Davies, 2001). AFPs type II, III, and IV appear to have arisen by a common mechanism whereby the duplication of a gene sequence of an existing protein or a protein domain occurs, followed by sequence divergence followed by acquisition of a new function. Not all APs have sequence similarities to currently known genes in the DNA databases and they are the type I helical coils in the flounder and shorthorn sculpin and the northern cod AFGPs and thus their ancestors are at present unknown. Despite their distinct origins and structures, all antifreeze proteins inhibit ice growth in a similar manner and can be pointed to as an excellent example of functional convergence.

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Overwintering in Submerged Turtles

DONALD C. JACKSON

Brown University, Department of Molecular Pharmacology, Physiology, and Biotechnology
Providence, RI, USA

Abstract. Freshwater turtles in cold latitudes overwinter underwater and may not have air-access for many weeks. If water is aerated, adequate O₂ can be obtained by nonpulmonary pathways to minimize or even avoid reliance on anaerobic metabolism. Certain species, such as the painted turtle, *Chrysemys picta*, are often found in ponds or swamps that become anoxic. These turtles can survive several months of experimental anoxia at low temperature, using a suite of adaptive traits that include hypometabolism, effective buffering utilizing their large mineralized shell, and the capacity for the usually hypoxia-intolerant organs, brain and heart, to function while anoxic.

Introduction

Turtles living in cold latitudes exhibit a variety of adaptations for winter survival. A single species, the painted turtle (*Chrysemys picta*), exhibits the extremes of these adaptations. As a hatchling overwintering in its natal nest, it can survive body temperatures as low as -10°C ; as an adult, this turtle can survive several months of submergence in anoxic water, relying strictly on anaerobic metabolism. Between these extremes are turtles that overwinter in burrows or buried under leaf litter, or that select aquatic habitats that are dependably oxygenated where they can extract the oxygen they require directly from the water (Ultsch, 1989). Because turtles are ectotherms, their body temperatures conform closely to their local environment, and therefore during long cold winters these animals are continuously at low body temperature and low metabolic rate.

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This paper will discuss turtles spending the winter submerged in water, and will focus on those species that live in climates where ice covers the water for weeks or months at a time. During periods of ice cover, submerged turtles are unable to breathe and must rely on nonpulmonary surfaces for any gas exchange. Even though their integuments are not particularly well-designed for exchange, most species at winter temperatures can supply all or much of their oxygen requirements if aquatic PO_2 is at ambient air levels, and can effectively excrete CO_2 to the water. A decrease in oxygen availability requires the turtles to shift to anaerobic glycolysis with lactic acid production. Most of the physiological information we have is derived from experimental submergences under simulated winter conditions in the laboratory, although several field studies have been conducted in which data have been obtained from animals overwintering beneath the ice. In addition, many field observations have documented the behavior of the various species (Ultsch, 1989).

Comparative studies of various North American turtles have revealed that species differ significantly both in their ability to obtain O_2 directly from the water and in their ability to survive under anoxic conditions. In general, the two traits are negatively correlated in that effective aquatic gas exchange is associated with poor anoxia tolerance, and conversely (Reese et al., 2002). Thus, the softshell turtle, *Apalone spinifera*, the musk turtle, *Sternotherus odoratus*, and the map turtle, *Graptemys geographica*, can all fully supply their oxygen requirements by aquatic respiration in well-aerated water at 3° C but are relatively poor at surviving submergence in anoxic water at this temperature. In contrast, the painted turtle, *Chrysemys picta* spp, and the snapping turtle, *Chelydra serpentina*, are less able to supply their O_2 needs by aquatic respiration, but are well-adapted for anoxic survival; indeed, the painted turtle is the most anoxia-tolerant vertebrate tetrapod known. Not surprisingly, the anoxia-intolerant species select hibernacula that are dependably oxygenated whereas the painted and snapping turtles are often found in ponds or swamps that can become hypoxic.

Submergence in Aerated Water

At summer temperatures, freshwater turtles such as painted turtles or sliders rely largely on their lungs for gas exchange. Much of their time is spent submerged and they come to the surface periodically and engage in a bout of breathing. This intermittent pattern maintains the animal in an aerobic state although the excursion of lung and blood gases above and below their mean values is more than is common in mammals. With the exception of certain species such as the

softshell turtle, turtles at warm temperatures exchange very little O_2 and only a modest fraction of their CO_2 via nonpulmonary avenues (Jackson et al., 1976). As temperature falls, however, metabolic requirements decrease considerably faster ($Q_{10} > 2$) than does gas diffusing capacity ($Q_{10} \sim 1.1$), so that for some species low temperatures are reached where aquatic respiration can satisfy O_2 needs and breathing is no longer required. For example, experimental submergences of softshell turtle (Reese et al., 2003) and map turtle (Reese et al., 2001) in aerated water at $3^\circ C$ for up to 150 days resulted in no significant change in plasma lactate in either species. This finding was verified in a field study of the map turtle (Crocker et al., 2000b).

In both the softshell turtle and the map turtle, blood PCO_2 fell during the submergence period, leading to a respiratory alkalosis. The reason for this was not established, but it could be due to enhanced aquatic gas exchange capacity in the hypoxic situation and/or to metabolic depression associated with submergence, as has been demonstrated in the frog *Rana temporaria* (Boutilier et al., 1997). Freshwater turtles have several possible avenues of extrapulmonary gas exchange: diffusion through the body surface, pumping water back and forth across the buccopharyngeal surfaces, and pumping water in and out of the cloaca and cloacal bursae (inflatable sacs that open off the cloaca). The last mechanism is exhibited most dramatically in the Australian species, *Rheodytes leukops*, in which cloacal bursae are highly vascularized and are a major site of gas exchange even at warm temperatures (Priest and Franklin, 2002). In North American emydids such as *Chrysemys picta* and *Trachemys scripta*, the sacs are simple structures with modest vascularization and are thought to function more in water storage or buoyancy control (Jackson, 1969; Peterson and Greenshields, 2001). The body surface of emydid turtles, such as the painted turtle, consists of keratinized skin and shell and seems better suited to resist exchange than to favor it; however, recent work (Rauer et al., 2002) indicates that the integument is the major avenue of O_2 uptake in painted turtles when submerged at $10^\circ C$. Even at $3^\circ C$ in aerated water, however, the aquatic gas exchange of the painted turtle is not adequate to supply all its O_2 requirements, and blood lactate concentration rises moderately during submergence (Jackson et al., 2000). It is not clear how the map turtle, a species of similar size and integument to the painted turtle, is able to satisfy fully its O_2 needs under these same conditions (Reese et al., 2001).

Submergence in Anoxic Water

Field observations have revealed that painted turtles are often (Ultsch, 1989) but not always (Crocker et al., 2000a) found buried in mud at the bottom of frozen ponds or streams. Bottom mud, because of its rich organic composition, is generally anoxic, so a buried turtle will have no access to oxygen. In addition, snow-covered ponds with little or sunlight may become hypoxic or even anoxic due to O₂ depletion by resident organisms. Severe hypoxia poses an environmental challenge that is intolerable for most aquatic vertebrates, but freshwater turtles can endure for many months under these conditions.

Three general traits can be identified that largely account for the painted turtle's remarkable anoxia tolerance: (1) hypometabolism greatly slows the rate of substrate depletion and the rate of end-product accumulation; (2) supplemental buffering by shell and skeleton and CO₂ loss to the water minimizes acid-base disturbance, and (3) maintained function of all organ systems, including the usually anoxia-intolerant brain and heart, despite anoxia and acidosis. These traits contribute to this animal's capacity to survive anoxic submergence at 3° C lasting three to five months (Herbert and Jackson, 1985a; Ultsch and Jackson, 1982). It is important to note the interdependence of these properties. Hypometabolism is a key element in permitting brain and heart function and it also slows the development of acidosis. Controlling the fall in pH is critical for maintaining a viable environment for organ function, and the moderate acidosis that does occur may help induce hypometabolism (Lutz, 1989).

Hypometabolism

Anoxic submergence of turtles results in a profound fall in metabolic rate, on the order of 80–90% (Jackson, 1968; Herbert and Jackson, 1985b). The animals in this state at low temperature remain motionless for long periods of time, but they do move about to some extent and are not completely comatose as has been suggested (Nilsson, 2001). Heart rates are greatly reduced to as little as one beat every several minutes (Herbert and Jackson, 1985b). Estimates of metabolic rate of anoxic turtles at 3° C, based on the accumulation and distribution of lactate, the principle anaerobic end-product, indicate a rate of 0.01 cal kg⁻¹ min⁻¹ (0.04 J). To put this value into context, consider that the resting metabolic rate of a similar-sized euthermic rat is about 100 cal kg⁻¹ min⁻¹, some four orders of magnitude higher. This huge difference in metabolic rate can be attributed to three factors: first, the turtle is an ectotherm and even at the same body temperature as the rat has a rate some 5–10 times lower; second, the reduction in

body temperature of the turtle from 37° C to 3° C further reduces metabolism by some 100-fold, an effect magnified by particularly high Q_{10} values in the low range of temperatures (Herbert and Jackson, 1985b); third, anoxia per se leads to an approximately 10-fold reduction as already noted. These effects are illustrated in Fig. 1.

The cellular basis for anoxia-induced metabolic depression is not well understood, but experimental evidence identifies effects both on ATP production via alteration in glycolytic enzymes (Storey and Storey, 1990; Storey, 1996) and on ATP utilization by downregulation of energy-requiring cell functions, such as ion pumping and protein synthesis (Hochachka et al., 1996). A key proposed alteration in the reduction in ATP utilization by ion pumps is a reduction in the leakage pathways of ions: what has been termed “channel arrest” (Hochachka et al., 1996). The reductions in ATP synthesis and hydrolysis are coordinated

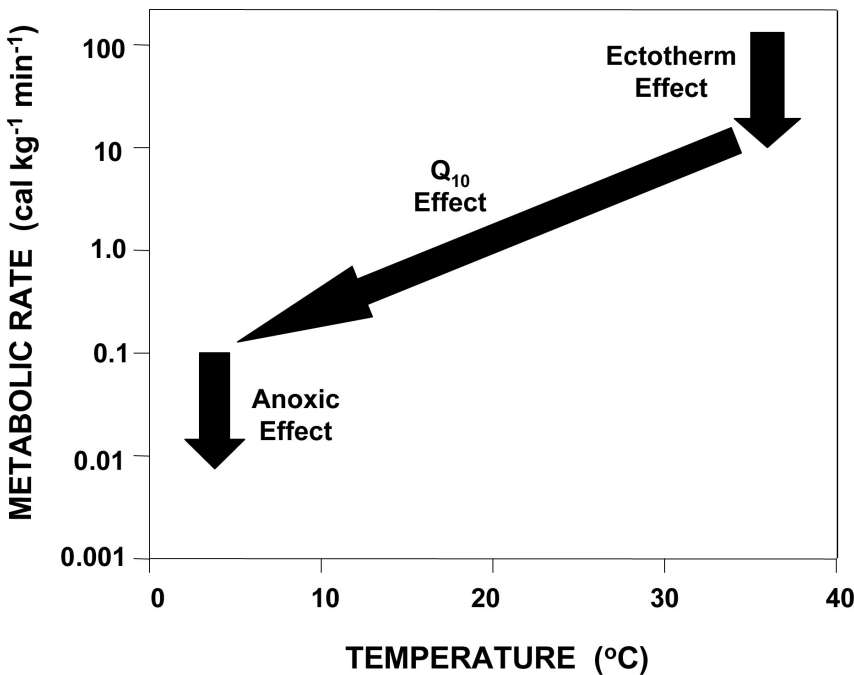


Fig. 1. This diagram illustrates the factors that contribute to the drastic reduction of metabolic rate of an anoxic cold turtle compared to a resting euthermic mammal. Modified from Jackson, 2000b.

so that cell ATP concentrations and energy state remain relatively unchanged during anoxia (Kelly and Storey, 1988). The role of acidosis, and in particular CO_2 retention, has been suggested to be a contributing factor to metabolic depression in mammalian hibernators (Malan, 1986), and may play a role in anoxic turtles (Lutz, 1989). As mentioned above, the most anoxic turtles are also the species with poorer extrapulmonary gas exchange and therefore generally higher PCO_2 values, especially early in submergence (Jackson et al., 2000; Reese et al., 2003). It is possible that the hypercapnic component to their acidosis depresses metabolism further and contributes to their anoxic tolerance.

Acid-base Balance

Despite the fall in metabolic rate, lactate rises to very high concentrations in turtles after three months or more of anoxic submergence, from normal plasma concentrations of about 1 mmol l^{-1} to $150\text{--}200 \text{ mmol l}^{-1}$, values that far exceed the available extracellular buffering power of turtles or other vertebrates. Nevertheless, blood pH under these conditions remains above 7.0 (normoxic pH at 3°C is ~ 8.0) and the strong ion difference (SID) of the blood (Stewart, 1981) remains positive.

The first line of defense against an acid load is the intrinsic buffering of the intra- and extracellular fluids. Intracellular buffering by turtles is not remarkable compared to other vertebrates (Shi et al., 1997), but extracellular (ECF) buffering is unusually high ($[\text{HCO}_3^-] \sim 40 \text{ mmol l}^{-1}$) in turtles such as the painted turtle and slider (Herbert and Jackson, 1985a). Of even greater importance, however, is the supplemental buffering contributed by the turtle's shell and skeleton (Jackson, 2000a). These mineralized structures participate in two ways: first by releasing calcium and magnesium carbonate into the blood and second by sequestering and buffering the lactic acid within the bone itself.

Release of carbonate buffers from bone is well known from other organisms, including man (Irving and Chute, 1932). In the turtle, however, because its renal function is largely shut down, the released calcium and magnesium, rather than being excreted to maintain normal plasma concentrations, accumulate in the ECF during anoxia, to as high as $50 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ and $15 \text{ mmol l}^{-1} \text{ Mg}^{2+}$ (Jackson and Ultsch, 1982), but about two thirds of the cations are complexed with lactate as CaLact^+ and MgLact^+ (Jackson and Heisler, 1982). Ionized Ca^{2+} is still very high, but cardiac function continues (Herbert and Jackson, 1985b) and brain intracellular $[\text{Ca}^{2+}]$ is defended (Bickler, 1998). *In vitro* studies of shell incubated in acid solutions simulating *in vivo* conditions indicate that bone

phosphate does not participate in the buffering process (Jackson et al., 1999) and that carbonate, which is particularly high in painted turtle shell (Jackson et al., unpublished observations), is the buffer anion.

Lactic acid also moves into shell and skeletal bone where it is buffered and stored, presumably largely complexed with calcium (Jackson, 1997). This is a novel mechanism not previously described that can account for over 40% of the total body lactate. It occurs in other organisms during lactic acidosis (Jackson et al., 2001; Jackson et al., 2003) but the importance is exaggerated in the turtle due to its large shell and high lactate levels. Together, the two bone mechanisms account for about 75% of the total lactic acid buffering in the turtle.

Brain and Heart

Studies of these normally vulnerable organs reveal that they are able to continue functioning during anoxic conditions albeit at a reduced metabolic level. Brain electrical activity is reduced and ion channels are down-regulated (Lutz and Nilsson, 1997) and the deleterious increases in intracellular calcium observed in anoxia-intolerant brains are avoided by inhibiting NMDA receptors and protecting intracellular calcium (Bickler, 1998; Bickler and Buck, 1998). Heart function is reduced but output is still adequate to meet the lower demands of the hypometabolic animal (Herbert and Jackson, 1985b). Isolated working hearts perfused with anoxic Ringer's solution maintain near normal cardiac output and cellular ATP levels, although when anoxia is combined with acidosis as occurs in vivo, cardiac function is more severely depressed (Wasser et al., 1990). Most importantly, both of these organs, as well as the rest of the body systems, are able to regain full function when O₂ is restored, as occurs normally in the spring (Herbert and Jackson, 1985a).

Conclusions and Future Directions

Freshwater turtles, unlike hibernating mammals or birds, overwinter underwater and may spend months without breathing. Anoxia-intolerant species, such as softshell turtles, must select sites that are dependably aerated, whereas anoxia-tolerant species, such as the painted turtle, can exploit sites that become hypoxic. The painted turtle, the most anoxia-tolerant vertebrate tetrapod studied, uses bone buffering and metabolic depression to manage anoxic durations and acid loads that are not possible for other tetrapods.

Our understanding of many aspects of these phenomena is still incomplete. For example, additional field work is required to document the extent to which

painted turtles and other species actually experience anoxia. Also, we are just beginning to learn how hatchlings and other pre-adult turtles survive their first winters of submergence and how anoxia tolerance changes with growth and development. In terms of basic mechanisms, much remains unknown about the trigger(s) for anoxic hypometabolism in the turtle and the metabolic coordination that permits profound reductions in both ATP production and utilization with little effect on cellular energy status. Finally, the cellular and/or chemical processes underlying buffer release from and lactic acid uptake by skeleton and shell in anoxic turtles are still uncertain.

Because of the extreme nature of its adaptations, the anoxic turtle has and should continue to serve as a model system for the study of many aspects of metabolic and acid-base physiology.

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Environmental Physiology of Terrestrial Hibernation in Hatchling Turtles

PATRICK J. BAKER, JON P. COSTANZO, AND RICHARD E. LEE, JR.
Miami University, Department of Zoology, Oxford, Ohio, USA

Abstract. Hatchling turtles hibernate either under water or on land. In northern regions, those hibernating within the frost zone must cope with desiccating conditions and extreme cold. Terrestrial hibernators exhibit several morphological, physiological, and behavioral adaptations to conserve water and to tolerate or avoid freezing. Here we review the environments inhabited by hatchling turtles and the strategies they use to overcome the challenges of life in the cold.

Hibernation Habitats and Winter Microenvironment

In late spring and early summer, female turtles deposit their eggs in shallow nests, excavated in suitably friable soils. The embryos complete development in 60 to 90 days and hatching occurs in late summer. Before the advent of winter, the hatchlings of many aquatic species emerge from their nests and, like adults, hibernate in thermally buffered aquatic habitats (Ultsch, 1989). Terrestrial hibernation, however, may enable hatchlings to reduce their risk of predation at a time when resources necessary for rapid growth are in decline (Wilbur, 1975). Field and laboratory studies have provided support for the model of Gibbons and Nelson (1978), which predicts that species that emerge in the fall forgo the benefits of terrestrial hibernation because they are unable to survive harsh winter conditions in the nest (Obbard and Brooks, 1981; Packard et al., 1993; 2000; Costanzo et al., 1995, 2000b, 2001b; Sims et al., 2001). Nevertheless, the offspring of a few northern turtles hibernate on land, either inside the natal nest or in the soil column below (Costanzo et al., 1995). Much of the research on the subject of hibernation in hatchling turtles has focused on species that

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hibernate inside the natal nest, since these species presumably encounter the most challenging of environmental conditions (but see D. Jackson's chapter in this volume). Variation in the hydric and thermal regimes among nests during embryonic development has long been known to influence the gender (Vogt and Bull, 1984) and size (Packard and Packard, 1988) of the resulting hatchlings. However, recent study (Costanzo et al., 2004) has shown that microenvironmental conditions within hibernacula at the same locale also vary considerably. Thus, nest-site selection by mother turtles may be an important determinant of winter survival (Tucker and Paukstis, 1999). Hatchling turtles that overwinter within the nest chamber require adaptations to limit water loss and, in some cases, to tolerate exposure to subfreezing temperatures (Costanzo et al., 2001b).

Water Conservation

Desiccation may adversely impact terrestrial hibernators because precipitation commonly falls as snow and because the water potential of the frozen soil matrix is low (Costanzo et al., 2001b). In addition, hatchlings are particularly prone to evaporative water loss because their surface area is large relative to their body mass (Mautz, 1982; Nagy et al., 1997). Morphological and physiological mechanisms of water conservation therefore may be critical to winter survival.

Low ambient temperatures and a reduction in metabolic rate may serve to reduce water loss in hibernating turtles (Gregory, 1982). Most terrestrial hibernators are highly resistant to dehydration, whereas species that typically hibernate in aquatic habitats lose body water via evaporation from body surfaces relatively easily when held in conditions of low relative humidity (Costanzo et al., 2001b). Although hatchlings apparently are unable to absorb moisture from their surroundings (Costanzo et al., 2001b), desiccation-resistant species conserve water by retaining urine (Costanzo et al., 2000b) and may offset any water loss (and perhaps even gain water) through oxidative metabolism of lipids and glycogenolysis (Wilson et al., 2001; Costanzo et al., 2004). Nevertheless, terrestrially hibernating hatchlings may dehydrate significantly during exceptionally dry winters (Costanzo et al., 2004). The relative importance of desiccation as a winter mortality factor, however, has not been determined.

Cold Hardiness: Freeze Tolerance and Supercooling

Freeze Tolerance

Extreme cold is another challenge confronting some terrestrial hibernators, as hibernaculum temperatures may occasionally fall below the equilibrium freez-

ing point of their body fluids, ca. -0.6°C (Costanzo et al., 1995; DePari, 1996; Packard et al., 1997; Nagle et al., 2000). One strategy to cope with extreme cold is freeze tolerance (i.e., survival of somatic freezing). Some turtles are well adapted to survive somatic freezing and can tolerate the freezing of more than half of their body water (Churchill and Storey, 1992a). To date, freeze tolerance has been demonstrated in hatchling *Chrysemys picta* (Storey et al., 1988), *Emydoidea blandingii* (Packard et al., 1999), *Malaclemys terrapin* (Baker et al., manuscript in preparation), *Terrapene ornata* (Costanzo et al., 1995), and *Trachemys scripta* (Churchill and Storey, 1992b), as well as adult *C. picta* (Claussen and Kim, 1993), *Terrapene ornata* (Costanzo et al., 1995) and *Terrapene carolina* (Costanzo and Claussen, 1990). These findings support Ultsch's (1989) hypothesis that freeze tolerance may be common among the Emydidae, a family of mostly North American pond turtles, although recent work suggests that one emydid, the northern map turtle (*Graptemys geographica*), appears to be freeze intolerant (Baker et al., 2003). Freeze tolerance has been examined in taxa within other families (i.e., Kinosternidae, Trionychidae, Chelydridae; Costanzo et al., 1995), but only hatchling *Chelydra serpentina* (Chelydridae) tolerate even modest freezing (Packard et al., 1993; Costanzo et al., 1995). Collectively, these findings cast doubt on the tenuous assertion that freeze tolerance is a common attribute among hatchlings of all species of turtles (Packard et al., 1999; Packard and Packard, 2001).

Turtles capable of surviving periods of somatic freezing must be specially adapted to cope with osmotic and ionotropic perturbations, ischemic anoxia, and metabolite end-product accumulation, which can contribute to freezing-related injury (Storey et al., 1988). The rapid accumulation of lactate in the brains of *C. picta* and *T. scripta* suggests that freezing induces acute anoxic stress (Hemmings and Storey, 2000) and therefore anoxia tolerance may be fundamental to freeze tolerance (Greenway and Storey 1999). There are limitations to this strategy since hatchlings of freeze-tolerant species tolerate somatic freezing only so long as their body temperature remains above ca. -4°C (Storey et al., 1988; Costanzo et al., 1995; Packard et al., 1999).

Freeze Avoidance by Supercooling

Another survival strategy used by hatchling turtles is freeze avoidance by sustaining a state of supercooling. Lacking freeze tolerance, hatchlings that routinely encounter subzero temperatures must rely on freeze avoidance. In order to supercool extensively, hatchlings must eliminate endogenous and ingested

ice-nucleating agents (INAs) that would otherwise seed the freezing of body fluids. Purging of INAs occurs during acclimatization to winter conditions (Costanzo et al., 2003) and is critical to the seasonal development of cold hardiness (Costanzo et al., 2000b). Generally, hatchlings can supercool extensively (from -8° to -20° C), owing in part to their small size (Costanzo et al., 2001b), if they manage to remain free of INAs.

A survival strategy based exclusively on freeze avoidance also has limitations. Since supercooled fluids are metastable, ice nucleation in turtle tissues can occur spontaneously or may be triggered by contact with ice and INAs in the environment (Costanzo et al., 2000a). It has long been presumed that ectotherms can recover from supercooling to low temperatures without injury (e.g., Spellerberg, 1972; Costanzo and Lee, 1995). However, recent studies (Packard and Packard, 1999; Hartley et al., 2000; Costanzo et al., 2001a) have shown that prolonged supercooling or chilling to very low temperatures causes physiological stress or even mortality. Owing to diminished tissue perfusion, survival in extreme cold probably requires a capacity for sustained anaerobic respiration as well as a tolerance for the decrease in pH associated with lactate accumulation (Hartley et al., 2000; Costanzo et al., 2001a; Baker et al., 2003). Anoxia-intolerant species may be constrained in their use of supercooling as a winter survival strategy, but this hypothesis has not been tested empirically.

Inoculation Resistance

Supercooling is a viable strategy of winter survival only if hatchling turtles are able to remain unfrozen during subzero chilling episodes (Lee and Costanzo, 1998). This is a particularly significant challenge confronting terrestrially hibernating turtles because the soil surrounding them teems with various INAs, such as soil particles, dust, and ice-nucleating microorganisms, which can inhibit supercooling (Costanzo et al., 2000a, 2001c, 2004; Baker et al., 2003). In addition, contact with ice in the winter microenvironment can readily trigger the freezing of supercooled turtles (Packard and Packard, 1993a; Costanzo et al., 1998).

Several studies (Costanzo et al., 1998, 2001c, 2004; Baker et al., 2003) have shown a strong association between soil characteristics and susceptibility to inoculative freezing (Fig. 1). Soil moisture is an important factor because it influences the probability that a hatchling will come into contact with ice in the soil matrix. For the same reason, susceptibility to inoculative freezing is influenced by characteristics of the soil such as particle size, porosity, and adsorptive capacity (Costanzo et al., 1998, 2001c). Intuitively, experimental tests of a species'

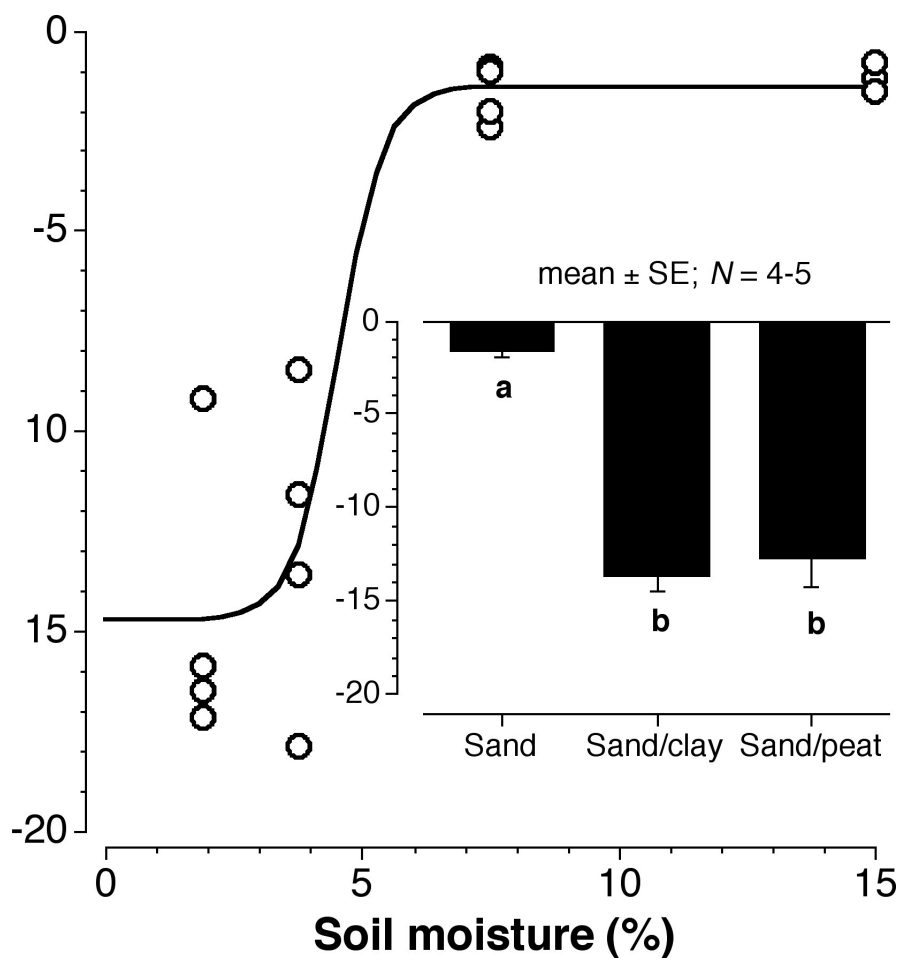


Fig. 1. Effect of soil characteristics on inoculation resistance, as indicated by the temperature of crystallization (T_c) of hatchling painted turtles (*Chrysemys picta*) cooled in a matrix of frozen soil. Response indicates the effect of variable moisture content in native loamy sand and the addition of 10% (w/w) clay or peat to native soil (inset; mean values identified by different letters were statistically distinguishable, $P < 0.05$). Adapted from Costanzo et al., 1998.

capacity for inoculation resistance should use native substrata and ecologically appropriate moisture levels; not doing so (e.g., Packard and Packard 1993a, 1993b, 1995) has led to some erroneous conclusions.

Capacity to resist inoculation by external INAs does not vary appreciably among populations of the same species (Costanzo et al., 2001c), but it is considerably greater in species that hibernate terrestrially than in aquatic hibernators (Costanzo et al., 2001b). Taxonomic variation in this trait may reflect differences in the amount of skin exposed to the environment (Costanzo et al., 2001b) as well as differences in the ultrastructure of the integument (Willard et al., 2000). For example, superior inoculation resistance in hatchlings of the painted turtle (*Chrysemys picta*) has been attributed to the presence of large deposits of unsaturated lipids in the dermal and epidermal layers of skin that is chronically exposed to the environment (Willard et al., 2000). Hatchling *G. geographica* also resist inoculation at low temperatures (Baker et al., 2003), but whether this

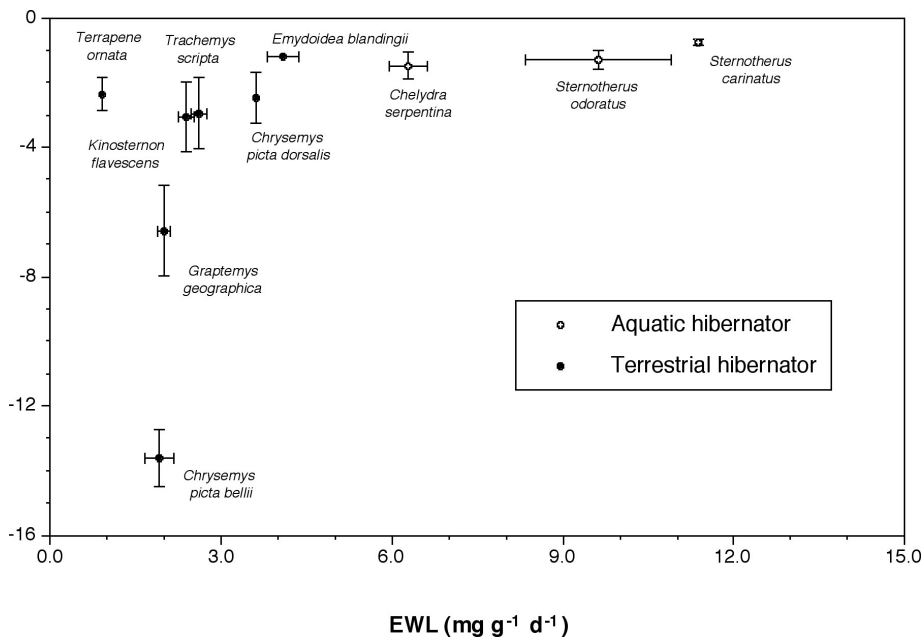


Fig. 2. Relationship between susceptibility to ice inoculation, as indicated by the temperature of crystallization (T_c), of hatchling turtles ($n = 4-9$) cooled in a matrix of frozen soil, and the rate of evaporative water loss (EWL; $n = 3-10$). Means are shown ± 1 SEM. Adapted from Costanzo et al., 2001b.

species also possesses an abundance of integumental lipids remains to be determined. Generally, species adept at resisting inoculative freezing also tend to have low rates of evaporative water loss, perhaps because a similar morphological attribute governs both properties (Fig. 2). This association attests that the integument plays a major role in adaptation to terrestrial hibernation (Costanzo et al., 2001b).

Conclusions

Despite the length and severity of winter in temperate North America, this region is populated by a diverse assemblage of semiaquatic and aquatic turtles. The hatchlings of a few species overwinter terrestrially and may experience desiccating and subfreezing temperatures. The survival of these turtles on both local and regional scales may be influenced primarily by nest temperature, precipitation, and the physical and hydric characteristics of nest soil that influence freezing risk (Costanzo et al., 2001c, 2004). At least one species, the painted turtle (*C. picta*), reportedly exhibits both freeze tolerance and a well-developed capacity for supercooling and may utilize either strategy, depending on physiological and environmental conditions.

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Overwintering in Tegu Lizards

DENIS V. ANDRADE,¹ COLIN SANDERS,^{1,2} WILLIAM K. MILSOM,² AND AUGUSTO S. ABE¹

¹Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, SP, Brasil

²Department of Zoology, University of British Columbia, Vancouver, BC, Canada

Abstract. The tegu, *Tupinambis merianae*, is a large South American teiid lizard, which is active only during part of the year (hot summer months), spending the cold winter months sheltered in burrows in the ground. This pattern of activity is accompanied by seasonal changes in preferred body temperature, metabolism, and cardiorespiratory function. In the summer months these changes are quite large, but during dormancy, the circadian changes in body temperature observed during the active season are abandoned and the tegus stay in the burrow and allow body temperature to conform to the ambient thermal profile of the shelter. Metabolism is significantly depressed during dormancy and relatively insensitive to alterations in body temperature. As metabolism is lowered, ventilation, gas exchange, and heart rate are adjusted to match the level of metabolic demand, with concomitant changes in blood gases, blood oxygen transport capacity, and acid-base equilibrium.

Seasonality and the Tegu Life Cycle

As with any other ectothermic organism, the tegu lizard, *Tupinambis merianae*, depends on external heat sources to regulate body temperature. Although this type of thermoregulatory strategy conserves energy by avoiding the use of metabolism for heat production (Pough, 1983), it requires that the animal inhabit a suitable thermal environment to sustain activity. When the environment does not provide the range of temperatures that enables the animal to be active year round, many species of ectothermic vertebrates become seasonally inactive (Gregory, 1982). Such a strategy is widespread amongst amphibians and reptiles

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of South America, although it is not always clear whether dormancy occurs in response to low temperature or to the accompanying dryness (Abe, 1995). The main landscape in Brazil, south of the Amazon, is characterized by savannah-like regions, called "Cerrado," alternating with tropical forests (Hueck, 1972). A wide belt of Cerrado crosses South America diagonally from northeastern Brazil to northern Argentina (Ab'Saber, 1977). The Cerrado is characterized by a seasonal climate consisting of alternations of cold, dry months (winter) with hot, rainy months (local summer) (Nimer, 1989). These cycles of rain and temperature dictate the annual pattern of activity for most species of amphibians and reptiles inhabiting the Cerrado (Abe, 1995).

The distribution of the tegu lizard, *T. merianae*, largely overlaps that of the Cerrado formation. Tegus are the largest members of the teiid family, attaining as adults a body length of up to 1.6 m and a body mass of up to 5 kg (Fig. 1). This lizard is an active forager, possessing powerful jaws and feeding on fruits, invertebrates, and small vertebrates (Vanzolini et al., 1980). In southeastern Brazil, tegus are active during the hot rainy season (approximately from August to April) and retreat into shelters, usually burrows dug in the earth, during the cold dry season (May to July) (Abe, 1983). From February onwards, activity and food intake decline markedly until the onset of the dormant season, at which



Fig. 1. An adult male tegu, Tupinambis merianae, pauses while foraging in southeastern Brazil.

time the lizards will retreat permanently into the burrow, occlude the entrance with plant debris, and stop food intake completely. After this time, tegus will not leave the burrow to bask, even on those occasional days when environmental temperatures attain levels equivalent to those seen in summer (Lopes and Abe, 1999). Thus, during dormancy, the marked diurnal cycles in activity, body temperature, and metabolism are abandoned. By the end of August to early September, tegus begin to emerge from dormancy, become progressively more active, and begin to bask and feed again. By this time, tegus in captivity will feed every other day and will gain weight, quickly recovering the losses incurred during dormancy. Breeding takes place approximately one month after the tegus have emerged from dormancy, when pairs are often observed in courtship and mating. By mid-October, females are gravid with extended bellies and egg laying occurs. An adult female may lay from 21 to 52 eggs; with the mass of the clutch being equivalent to 40% of the female's post-partum body mass (see Lopes and Abe, 1999, for more details on the reproductive biology of tegus). Although this represents a large investment in reproduction, females are able to recover the weight loss during the remainder of the active season. During the months leading up to entrance into dormancy, the fatty mass stored in the abdominal cavity increases to about 5% of total body weight. It is estimated that this amount of fat is sufficient to sustain metabolism in dormancy for more than one full year (Abe, 1995).

Temperature and Metabolism

Over the course of a year, tegus show a variable thermal profile that is distinctive for each season (Fig. 2). While active in the summer, body temperature (T_b) rises rapidly in the morning with basking, slowly continues to increase during the day, and then gradually declines once the tegu re-enters the burrow in the evening. This pattern is common throughout the active season, although mean T_b appears to be higher and more variable (has a wider daily range) during spring and summer, than in fall. Interestingly, throughout the active seasons, the daily maximum for T_b is about 35–36° C, a value quite close to the regulated T_b of many endotherms. In the fall, at the onset of dormancy, T_b falls relatively quickly over a short, multiday period. At present, it is unclear whether this is the result of concomitant changes in the weather (e.g., several days of rain), or an active strategy employed by the animal to prepare for entrance into hibernation. During hibernation, the little variation in T_b that remains is assumed to reflect changes in the ambient temperature in the hibernaculum (see Fig. 2).

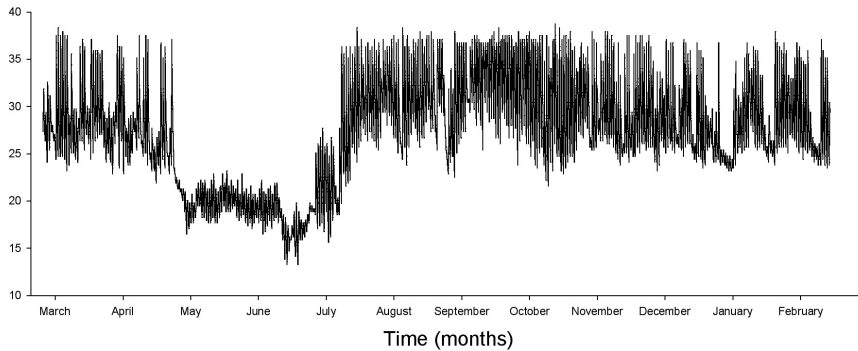


Fig. 2. Body temperature profile of an adult male tegu lizard (~3.5 kg) during one full year of recording with an implantable data logger. Dormancy is clearly distinguishable, from late April to late July. Multiday bouts of inactivity in the burrow can be seen from January until late April. (Recordings taken every 16 minutes, from Elis R. Lopes, unpublished data.)

During the active season, tegus become active each day, not with sunrise, but rather with increases in ambient temperature (Fig. 3). During the night, ambient temperature falls below the temperature of the burrow and the tegu. During the day, once the ambient temperature rises to equal that of the resting tegu, the lizard emerges from the burrow to bask. Basking induces a rapid increase in T_b ($\sim 2^\circ \text{C/hr}$ in an $\sim 3.5 \text{ kg}$ tegu) to a maximum T_b of $\sim 33\text{--}38^\circ \text{C}$. This is accompanied by parallel increases in metabolism, heart rate (from a resting value of ~ 10 beats/min to ~ 70 beats/min) and respiratory frequency (from a resting value of $\sim 2\text{--}3$ breaths/min to $\sim 12\text{--}18$ breaths/min). Once warm, the tegus become active and spend the day alternating between periods of basking, foraging, and resting in the burrow, behaviourally maintaining T_b between 32°C and 38°C . Later in the afternoon, the tegu re-enters the burrow to rest for the night. The rate of heat loss ($\sim 0.44^\circ \text{C/hr}$ for an $\sim 3.5 \text{ kg}$ lizard) throughout the night is slow and appears to be physiologically regulated. A dead tegu of similar mass, under the same environmental conditions, loses body temperature at a rate of $\sim 1.28^\circ \text{C/hr}$ (Colin Sanders, unpublished data).

During hibernation, tegus exhibit a depressed metabolism (Abe, 1983), although the nature and extent of this depression in nature are still unclear. Abe (1983) recorded a Q_{10} for metabolic rate (VO_2) of about 1.1, over a temperature range of $20^\circ\text{--}30^\circ \text{C}$ in a hibernating tegu recovered from the wild. This sug-

gested that an absolute level of metabolic suppression had been reached that was temperature independent. In a subsequent study in which hibernation was induced in tegus in captivity, Abe (1983) found a Q_{10} of 2.9 for VO_2 over the same range (20°–30° C). While this suggested that the absolute level of metabolic suppression was not temperature independent, metabolism was nonetheless suppressed compared to that of active tegus during the summer over the same temperature range (inverse acclimation) (Abe, 1995; Andrade and Abe, 1999). Thus, although increases in temperature produce increases in metabolism in dormant tegus, the magnitude of this increase is less than that exhibited by active tegus in the summer over the same temperature range.

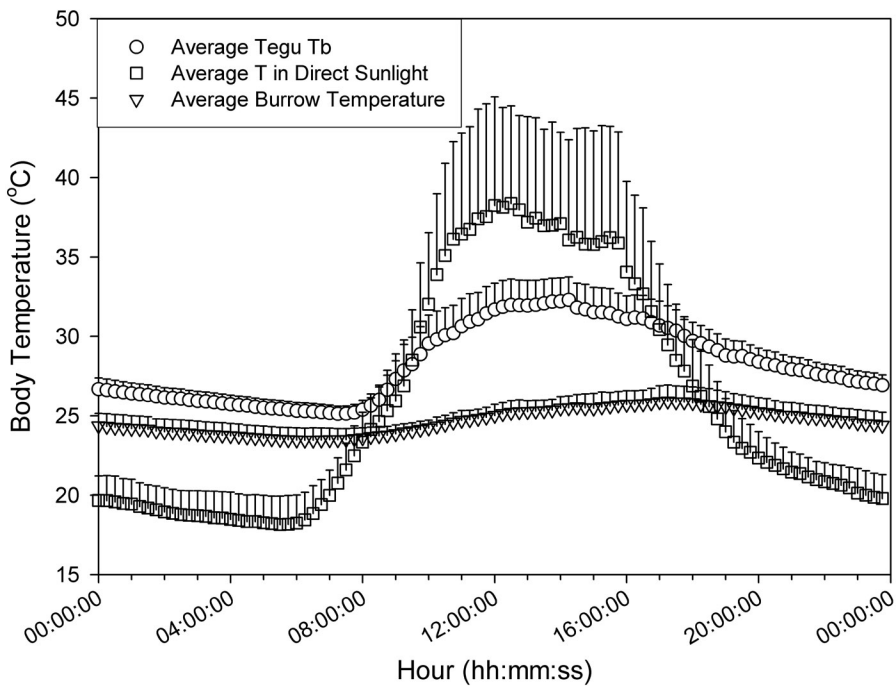


Fig. 3. The average daily thermal profile of a group of adult tegus ($n = 6$, average mass = 3.7 ± 0.33 kg), their burrows, and a data logger in direct sunlight, during days when tegus were active during the month of January 2004. Average values \pm SEM.

Cardiorespiratory Adjustments

The primary function of the cardiorespiratory system is to transport adequate amounts of O_2 from the external environment to the tissues and, conversely, remove the metabolically produced CO_2 from the tissues to the external environment (Hlastala and Berger, 2001). "Adequate" here means that the rate of O_2 delivery and CO_2 removal meet the metabolic demands of the animal and prevent the development of tissue hypoxia/anoxia and/or acidosis and their deleterious consequences. Given that the tegu lizard experiences marked seasonal changes in activity, body temperature, and metabolism, seasonal adjustments in cardiorespiratory function are also expected to occur.

As metabolism is depressed, animals require less O_2 and produce less CO_2 and thus lung ventilation decreases. While an active tegu usually breathes 10 ml/kg/min, a dormant tegu at the same temperature breathes 4 ml/kg/min (Andrade and Abe, 1999). In dormancy, tegus also exhibit a change in ventilatory pattern; at 17°C these lizards breathe episodically, with a few grouped breaths (five to six breaths) separated by periods of apnea as long as 26 minutes; the longer the duration of apnea, the greater the number of breaths in the subsequent breathing episode. O_2 extraction and CO_2 excretion occur at different rates during breath holding in dormant tegus; O_2 is extracted more rapidly from the lungs than CO_2 is eliminated (Andrade and Abe, 1999). As a result, lung volume changes during the breath-holding period, raising the possibility that a mechanical cue is involved in the regulation of the breathing pattern of dormant tegus. The differences in the dynamic of O_2 and CO_2 exchange may also explain why dormant tegus have elevated levels of total CO_2 in the blood, while O_2 content is almost unaltered (Andrade et al., 2004; see below).

Given that tegus do not move around during dormancy, the most conspicuous activity they perform during this period is breathing. Since all other activities shut down during dormancy, the relative cost of the remaining activity will be increased. Thus, it is not surprising that the oxidative cost of breathing of dormant tegus has been estimated to be 52.3% of the total metabolism (Andrade and Abe, 1999). This figure is even higher for hibernating squirrels breathing episodically, where the cost of breathing has been estimated to account for 90% of the total metabolism (Garland and Milsom, 1994). It has been suggested that the change to an episodic breathing pattern during dormancy might, due to mechanical considerations, represent a strategy to minimize the energetic costs of breathing, and this would appear to be a strategy shared by mammals and reptiles (see Milsom, 1991). Interestingly, however, if the temperature of

dormant tegus is increased to 25° C, their breathing becomes regular. In this situation, metabolism is somewhat elevated by the change in temperature, but this increase is accompanied only by an increase in total ventilation with no noticeable change in the air convection requirement. This matching of ventilation to metabolism is caused by reducing the duration of the apneic periods, i.e., by alterations of breathing frequency rather than changes in tidal volume (Andrade and Abe, 1999).

Recently, we (Andrade et al., 2004) extended these studies to address the question of whether the blood characteristics associated with the transport of the respiratory gases also change seasonally. Furthermore, considering that the seasonal adjustments in the cardiorespiratory system aim to maintain adequate levels of blood gases and acid-base equilibrium *in vivo*, we also wondered whether the definition of “adequate levels” might vary seasonally. We found that during dormancy, tegus exhibited a lower blood oxygen-carrying capacity than during the active season, reflecting the need to transport less O₂ to meet the lower metabolic rate typical of dormant tegus. The lower blood oxygen-carrying capacity observed in the dormant tegus was due to concurrent decreases in hemoglobin concentration ([Hb]) and hematocrit (Hct) and an increase in nonfunctional met-hemoglobin content. Dormancy caused blood–oxygen affinity in *T. merrianae* to increase independently of pH and temperature. Increased blood–oxygen affinity is a common feature of metabolic depression (Jokumsen and Weber, 1980; Maginniss and Milsom, 1994) and facilitates pulmonary O₂ loading. In the case of dormant tegus, the left-shift in the oxygen equilibrium curve (O₂EC) might be particularly important because a higher blood oxygen affinity would allow pulmonary O₂ extraction even during the final portion of extended periods of apnea (see also Lapennas and Lutz, 1982). At the same time, a high blood O₂ affinity decreases the capillary-to-tissue oxygen diffusion rate and promotes tissue hypoxia. This has been suggested to be a mechanism involved in the down regulation of aerobic metabolism (see Leggio and Morpurgo, 1968; Jokumsen and Weber, 1980). As reported for other ectothermic vertebrates, the arterial pH decreases with temperature in *T. merrianae*, while arterial O₂ partial pressure (Pa_{O₂}) and arterial CO₂ partial pressure (Pa_{CO₂}) increase. Arterial O₂ content (Ca_{O₂}) and CO₂ content in the plasma (Cpl_{CO₂}) are not altered (Glass et al., 1985; Stinner and Wardle, 1988; Wang et al., 1998). Dormancy is accompanied by an increase in plasma bicarbonate ([HCO-3]pl) and an elevation of Pa_{CO₂} and Cpl_{CO₂}, suggesting that, on a seasonal basis, tegus regulate arterial pH at the expense of changes in Pa_{CO₂} and [HCO-3]pl (see also Rocha and Branco,

1998). As a consequence, at most temperatures, arterial pH (pHa) does not differ between seasons. However, at low temperatures, tegus regulate pHa at a relatively acidic level, suggesting that tissue acidosis may also be involved in the down-regulation of metabolism in these lizards, as has been postulated to occur in other animals (Malan, 1993; Guppy et al., 1994; Boutilier et al., 1997).

Perspectives

Undeniably, the most complete picture of the physiological adjustments accompanying seasonal depression in metabolism has arisen from studies on mammals. However, the study of ectothermic vertebrates might provide a fruitful alternative for such studies, providing a system where one can potentially separate the consequences of altering metabolism and body temperature, since these two functions are not as intricately intertwined in ectotherms as they are in endotherms. Tegus are large animals, which facilitates instrumentation. They breed successfully in captivity, they grow rapidly, and they can be obtained in large numbers. So far, we have documented only a few aspects of the physiological adjustments associated with the metabolic depression experienced by this lizard. We have not even begun to examine the exciting topic of the cues used by this lizard to establish its seasonal cycle nor the proximal factors that trigger dormancy. The humoral, neural, genetic, and molecular basis of the seasonal pattern of activity in *T. merianae* remain unexplored aspects of "life in the cold," taking place south of the Amazon.

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Overwintering in Cold-Submerged Frogs

GLENN J. TATTERSALL

Brock University, Department of Biological Sciences, St. Catharines, Ontario, Canada

Abstract. Numerous physiological, biochemical, and behavioural adjustments take place that enable aquatic overwintering amphibians to survive the winter in an unfrozen state. These adaptations appear to represent an orchestrated hierarchy of defence mechanisms. Frogs exploit the variable temperature and oxygen levels of their overwintering environment, allowing them to continuously change their preferred microhabitats and thus minimise the stress associated with either hypoxia or hypothermia. When or if these behavioural strategies are insufficient to ensure survival, major physiological changes occur, including adjustments in anaerobic and aerobic capacities and efficiencies. This chapter will focus on the physiological and behavioural mechanisms available to those amphibians for which the most is known (i.e., the aquatic overwintering frogs of northern temperate climates).

Background

Many amphibians encounter large fluctuations in the physico-chemical nature of their ambient environments (e.g., temperature, salinity, and oxygen) that can seriously compromise their survival. On a yearly basis, temperature alone can vary from below 0° C to above 35° C in temperate species. Being ectothermic, amphibians could be considered to be at the mercy of these vast changes in their physical environment. However, it is well known that homeostatic mechanisms exist that help to minimise environmental stresses. Physiological mechanisms, such as aestivation (Guppy et al., 1994), metabolic reduction (Hand and Hardewig, 1996; Hochachka and Guppy, 1987), and temperature compensation (Clarke, 1993) are generally well studied. However, the role that the animal's be-

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haviour plays within these variable environments is often not considered, since most temperature studies focus on acclimation to constant environments (for references see Johnson et al., 1996). Ectotherms have the option of using naturally occurring spatial variations in temperature in order to maintain optimal body temperatures and thereby manipulate or maintain metabolic rates.

Amphibians are found in a diverse range of habitats around the globe, and temperate-zone amphibians occur further north than any other ectothermic tetrapods (Ulrich, 1989). The long, cold winters encountered by these amphibian species demands that a considerable amount of their lives is spent in a state of combined metabolic and/or temperature-induced torpor and starvation (Pinder et al., 1992), having to overwinter in some instances for periods of up to nine months (Bradford, 1983; Pasanen and Koskela, 1974). Because most of these temperate species cannot survive below 0° C, unlike the freeze-tolerant *Rana sylvatica*, they are unable to spend the winter above ground. This leaves these more aquatic ranid frogs no other choice but to spend the winter “trapped” and submerged under the ice cover of small ponds and lakes.

The Overwintering Environment

The aquatic environment under the ice cover of lakes and ponds undergoes drastic changes throughout the course of the winter. While the environment is uniform in temperature and, indeed, highly oxygenated in the late autumn, the limnological conditions change greatly once ice develops on the surface. The lack of convective mixing and the temperature-dependent density of water leads to a thermal gradient forming, such that waters vary from 4° C at the bottom to 0° C in the layer just below the ice. An additional effect of surface ice formation is a progressive decline in dissolved oxygen levels that ensues from the consumption of the resident organisms and the detritus. Photosynthetic production of oxygen cannot keep pace with oxygen consumption, resulting in a potentially lethal level of hypoxia. In severe cases, oxygen levels can fall dramatically, with only the shallowest areas retaining any oxygen at all, primarily by diffusion through the ice (Greenbank, 1945). The net result is that stratification for both oxygen and temperature occurs, with warm, hypoxic water on the bottom (4° C, <20 mmHg or <1.7 mg O₂ L⁻¹) and cold, partially oxygenated water just below the ice (0° C, 60–85 mmHg or 5–15 mg O₂ L⁻¹) (Bradford, 1983; Greenbank, 1945). This leaves the overwintering frog in a precarious situation where it must not only cope with the dwindling supplies of oxygen but also with a finite supply of energy stores in the form of fat and carbohydrate. The crucial

question then becomes one of how the frog stays alive by balancing its energy supply and demand with the ever-diminishing resource of oxygen; in some cases many do succumb, and massive die-offs (winterkill) are well documented. For example, populations of *Rana temporaria* have been known to crash by 97–100% (Bannikov, 1948; Pasanen and Sorjonen, 1994). Dense populations of *R. temporaria* have suffered massive die-offs (1,000 dead in one year and 700–800 dead the following year in a small pond; cf. Pasanen and Sorjonen, 1994) over the course of the winter. Even in clear alpine lakes, large numbers of adult frog deaths have been attributed to the severe hypoxia associated with prolonged ice cover (Bradford, 1983). It comes as no surprise that many of these frogs are considerably hypoxia-tolerant, given the large selection pressures that exist during the prolonged overwintering periods in their oxygen-poor environments.

Physiology of Overwintering Amphibians

Relatively little information was available until recently on the physiology of aquatic hibernators (animals that do not tolerate freezing) such as the common frog, *R. temporaria*, and the numerous North American ranids. Most invertebrates, and many vertebrates (including frogs) exhibit a reversible metabolic depression in response to environmental stress such as anoxia or hypoxia (Guppy et al., 1994). In addition to hypoxia, overwintering frogs are exposed to cold, which leads to further decreases in metabolism. To date, little documentation exists of the true Q10 values at low temperatures (0–5° C), which makes it difficult to discern the mechanisms overwintering frogs might exploit to suppress their metabolism. Do they suppress their metabolism via a coordinated reduction in cellular and organ processes, or do they exploit high Q10 values to reduce their metabolism? Evidence exists to support both; an overwintering metabolic suppression has been shown in both normoxic- and hypoxic-submerged frogs (Donohoe and Boutilier, 1998; Donohoe et al., 1998) and Q10 values from 1 to 5° C range from 4–6, (Dunlap, 1972; Tattersall and Boutilier, 1997), rather than the values of 2–3 typical of higher temperature ranges.

The ability to reduce metabolic demands and thereby conserve on-board fuels during overwintering episodes is thought to be a strategy employed by many such animals. Overwintering amphibians can improve their chances of surviving unpredictable winters by reducing their metabolic rate (Boutilier et al., 1997). This, in fact, is what happens in cold-submerged frogs in the laboratory during the overwintering period (Donohoe et al., 1998). Common frogs submerged at 3° C underwent a 61% suppression of aerobic metabolic rate. By reducing

metabolic rate to this extent, the total length of time required to completely oxidize the limited on-board fuel reserves would be extended from 90 to 150 days, allowing more than enough time to survive the winter (Boutilier et al., 1997).

Under the cover of ice, frogs are entirely aquatic and must therefore rely exclusively on cutaneous respiration for both oxygen uptake and carbon dioxide excretion. Cutaneous respiration is very advanced in amphibians (Feder and Burggren, 1985a), and at low temperatures is more than enough to accommodate their total metabolic requirements. The normal pattern for blood flow in air-breathing amphibians, where deoxygenated blood is shared between the lungs and the skin, changes entirely upon submergence. After only a few hours of submergence at low temperatures, all the oxygen in the lung becomes depleted, and oxygen uptake must then be accommodated entirely through the skin. This is almost certainly associated with a right-to-left intraventricular shunting of blood, a feature common to diving reptiles and amphibians (Wood, 1984) and one that may contribute to the hypoxia and anoxia tolerance so well established in these lower vertebrates (Hochachka, 1988).

When exposed to ambient hypoxia, oxygen delivery to certain tissues is drastically altered. Whereas blood flow to hypoxia-sensitive organs such as the brain and heart is maintained, perfusion is reduced to the bulk of the skeletal muscle and other less essential organs. However, the skin must continue to be perfused at a high rate in order to maintain oxygen uptake (Armentrout and Rose, 1971; Poczopko, 1957), since pulmonary oxygen uptake is no longer possible. Numerous studies on lungless salamanders suggest that skin-breathing amphibians are primarily diffusion limited for gas exchange (reviewed by Feder and Burggren, 1985b; Gatz et al., 1975; Piiper et al., 1976). As a result of this diffusion limitation, skin breathers are thought to be poor regulators of gas exchange, since increasing cutaneous blood flow is thought to be an ineffective means of increasing oxygen uptake when required. However, most of these experiments were conducted at higher temperatures, where skin perfusion is likely already at its maximum, so extrapolating to much lower temperatures may lead to erroneous conclusions about the effectiveness of cutaneous gas exchange in overwintering frogs (Burggren and Moalli, 1984; Pinder, 1987; Tattersall and Boutilier, 1999b). For example, cold-submerged bullfrogs can regulate cutaneous oxygen uptake either through spontaneous ventilation of the skin, thereby disrupting hypoxic boundary layers, or by the recruitment of otherwise under-perfused capillaries in the skin (Pinder, 1987).

Meanwhile, many tissues of hibernating frogs may enter into a hypometabolic state (as seen in aestivating frogs; Flanigan et al., 1991), thus reducing total energy expenditure. The ability of many ectotherms to exploit a reversible metabolic suppression is thought to be one of the most important physiological adaptations to hypoxia or anoxia. Donohoe and Boutilier (1998) demonstrated that when hibernating frogs encounter hypoxic conditions, they initially accelerate their use of glycogen reserves by recruiting anaerobic metabolism. However, they subsequently avoid a runaway lactacidosis by reducing overall metabolism. This so-called hypometabolic rescue of aerobic metabolism further conserves the animal's limited on-board substrates by enabling ATP production to match ATP consumption aerobically. This hypometabolic rescue may be key to their survival during unpredictable winters.

Mechanisms of Metabolic Suppression and Hypoxia Tolerance

When oxygen is limiting to tissues, as it is in submerged overwintering frogs, the metabolic depression is also accompanied by increases in metabolic efficiency, particularly in hypoxic environments. St-Pierre et al. (2000c) found that the isolated mitochondria from frog skeletal muscle undergo reductions in State 3 respiration and increases in efficiency (i.e., its affinity for oxygen) during four months of hibernation. Both normoxic and hypoxic submerged frogs exhibited increases in mitochondrial oxygen affinity; however, this increase was most pronounced during the first month of overwintering. This is likely due to the fact that whole animal metabolic rates can take months to readjust to lower levels, and thus the greatest increase in oxygen affinity occurs during the early, more intensely stressful period of overwintering. Further evidence for this is seen in the transient lactate accumulation in hypoxic submerged frogs, which disappears after one month of submergence (Donohoe and Boutilier, 1998).

Accompanying these changes in affinity are changes in other aspects of aerobic metabolism. For instance, the proton motive force (the membrane potential across the inner mitochondrial membrane), which dictates the movement of protons and ultimately determines the rate of ATP synthesis through oxidative phosphorylation, is reduced during overwintering (St-Pierre et al., 2000a). This indicates a decrease in the activity of the components of the electron-transport chain, possibly to conserve the limited on-board fuels. A further advantage of the reduced proton motive force is the consequent reduction in proton leak that occurs as a result of the lower membrane potential (St-Pierre et al., 2000a). So far, however, no evidence for a reduction in the actual mitochondrial proton leak

pathways has been found in amphibian tissues nor in any animal that undergoes significant metabolic suppression, suggesting a limitation on the reorganisation that can take place during aquatic hibernation.

Another potential change in efficiency of aerobic metabolism is related to the function of the F1F0-ATPase molecule itself. Normally, this protein is responsible for producing ATP by harnessing the potential energy stored in the proton motive force. However, during anoxia (a condition that may be transiently encountered in aquatic amphibians during the overwintering period), this protein becomes a net ATP consumer, rather than operating as a net ATP producer. When oxygen is in short supply, the electron transport chain is not functional and thus protons are not being transferred across the inner mitochondrial membrane to maintain the proton motive force (i.e., the mitochondrial membrane potential). By operating in reverse, the F1F0-ATPase can operate to maintain mitochondrial membrane potential. Overwintering frogs, however, are capable of modulating the rate at which this enzyme operates (St-Pierre et al., 2000b). Under anoxic conditions, the F1F0-ATPase operates at much more reduced levels from that under more highly oxygenated conditions, suggesting a modulatory capacity to this process. What this translates into in terms of energetics is a much reduced rate at which ATP is consumed in anoxic tissues; however the small turnover of ATP must still be sufficient to maintain mitochondrial membrane potential. Although it is not clear why this occurs, it is possible that maintaining the membrane potential can prevent mitochondrial damage and/or programmed cell death that would otherwise occur due to a collapsed membrane potential.

The biochemical adaptations possessed by amphibians for economising on fuel usage and maximising ATP provision seem adapted toward a time-dependent and stress-dependent response. This may be the best physiological design available to amphibians, since it is flexible enough to respond according to the length and severity of winter encountered.

Behaviour of Overwintering Amphibians

Changes in body temperature through behavioural thermoregulation may have an important role in allowing frogs to manipulate their own metabolism in order to enter into and out of a transient temperature-induced metabolic suppression. Frogs in the wild have long been assumed by naturalists to “hibernate” in an inactive state while burrowed in the mud bottom of ice-covered lakes and ponds (Hutchison and Dady, 1964; Pinder et al., 1992). However, field studies

conducted on radio-tagged bullfrogs reveal that substantial underwater movements occur throughout the winter that appear to demonstrate an avoidance of the anoxic mud and detritus (Stinner et al., 1994). Many overwintering habitats develop large spatial and temporal fluctuations in temperature and oxygen. As a result, one would expect that animals living in these complex environments would evolve behavioural mechanisms that optimise survival or, at the very least, avoid lethal extremes.

Field studies have shown that toward the end of winter, bullfrogs appear to select microhabitats very close to the ice, in the colder, shallower waters (Stinner et al., 1994). If this is true, then frogs may either be exploiting their potentially high Q₁₀ values so as to suppress metabolism by choosing lower temperatures, or they may be following oxygen gradients and selecting oxygen-rich waters near the surface. In addition, many amphibians exhibit a behavioural hypothermia response (i.e., the active choice of a colder ambient temperature), as a regulated means of lowering body temperature when exposed to an environmental stress such as reduced oxygen levels (Dupré et al., 1988; Hicks and Wood, 1985; Wood, 1995; Wood and Malvin, 1991). The resulting "hypothermia" reduces metabolic demands, increases blood oxygen saturation, and decreases the cardiovascular costs associated with hypoxia, thereby extending the survival time during periods of hypoxic stress (Wood, 1995). Tattersall and Boutilier (1997) showed that cold-submerged frogs can and do make behavioural "decisions" aimed at reducing metabolic costs during hypoxic stress. Upon exposure to hypoxic water, these cold-submerged frogs lower their preferred temperature by approximately 5° C, and as a result of this decline in body temperature, reduce their metabolic expenditure by over 70%. This decrease in overall metabolism prevents or reduces the requirement for anaerobic metabolism to make up for the shortfall in ATP requirements that aerobic metabolism can no longer provide. Despite the information provided from these laboratory experiments, we still do not know whether behavioural thermoregulation plays a major role in regulating metabolic costs within the natural overwintering environment.

A final confounding problem for amphibian behaviour during the winter is the risk of predation (Emery et al., 1972). Frogs cannot tolerate prolonged anoxia (Boutilier et al., 1997), so they are unlikely to burrow into the mud to avoid detection for more than a day or two. This suggests that submerged frogs are unable to completely disappear into the mud during the winter and instead must evade discovery by remaining active and selecting microhabitats that best support aerobic metabolism. Predation risk may disrupt or even prevent normal

thermoregulatory behaviour during the winter, and frogs that escape predation would still require long periods of time to fully recover. The role of behavioural thermoregulation following internally derived hypoxia and metabolic disturbances has recently been appreciated in ectotherms (Tattersall and Boutilier, 1999c), wherein frogs transiently prefer cooler temperatures after intense activity. The contribution of activity and exercise toward changing thermoregulatory behaviour during the winter is poorly appreciated and may play an important part in overwintering survival.

In terms of oxygen balance and acquisition, behaviour can be of great importance in maintaining body oxygen stores in amphibians. In submerged, overwintering amphibians, surfacing to breathe air is not an available option for maintaining oxygen uptake, so the behavioural detection of oxygen or temperature is the only immediate means by which they can adjust either the supply or the demand of oxygen and thus maintain metabolic homeostasis aerobically (Tattersall and Boutilier, 1999a). Further, these “decisions” must be made within the natural setting of the overwintering pond, where external influences such as environmental change, predation, and social aggregation may conflict with the ability of the frog to exhibit an ultimate environmental preference.

Concluding Remarks

One mystery regarding the integrative biology of overwintering amphibians is reconciling the fact that metabolic suppression can occur contemporaneously with behavioural changes in preferred temperatures and oxygen detection. How these animals reduce and restructure their physiological machinery sufficiently to minimise energetic costs and yet are still capable of movement, exercise, and responding to external stimuli is not known. Most other animals that enter into a reversible metabolic suppression exhibit major changes in behaviour (i.e., become behaviourally dormant or quiescent). Perhaps, as in many things, amphibians have adopted a middle of the road approach, rather than an all or none approach, not exploiting the benefits of a full-blown metabolic suppression. It is likely that these differences in physiological tolerances reflect the presence of behavioural responses and have shaped the overwintering biology of temperate amphibians.

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Effect of Temperature on Regular and Modified Circannual Rhythms in the European Ground Squirrel Under Free-Running Conditions

RADOSLAV K. ANDJUS,¹ MARINA MARJANOVIC,² AND DRAGOSLAVA ZIVADINOVIC³

¹ Center for Multidisciplinary Studies, University of Belgrade, Belgrade, Serbia-Montenegro (passed away in 2003)

² Eastern Illinois University, Department of Biological Sciences, Charleston, Illinois, USA

³ University of Texas Medical Branch, Human Biological Chemistry and Genetics, Galveston, Texas, USA

Abstract. In this long-term study (up to eight years), body mass (τ_{bm}) and hibernation/activity periods (τ_{hib}), and the cumulative phase shift of consecutive cycles were determined in the European ground squirrel (*Spermophilus citellus*) under free-running conditions at different ambient temperatures. At 15° C, average τ_{bm} (0.876 ± 0.019 years) was significantly longer than the period obtained at 30° C (0.826 ± 0.013 years); however, values at 30° and 7° C (0.796 ± 0.027 years) were not significantly different. These results confirmed the temperature dependence of the free-running period of circannual cycles in *S. citellus*, but they also showed that the effect of temperature on τ_{bm} could vary with the temperature range. τ_{bm} and τ_{hib} at the same temperature were not significantly different, verifying that body mass and hibernation/activity cycles were phase-locked. We also found drastically modified rhythms (“accelerated rhythm”—high frequency, short active phase; “protracted hibernation”—activity period less than seven days). Out of 98 cycles at low temperatures, three modified cycles were recorded at 15° C and 36 were recorded at 7° C. Modified rhythms at 7° C had a remarkably big negative phase shift of maximal body mass (212 days/cycle), indicating that more than two cycles can be completed in one year.

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Introduction

Hibernators exhibit endogenous circannual rhythms that persist in the absence of seasonal cues (see reviews: Pengelley and Asmundson, 1974; Mrosovsky, 1978). When exposed to constant environmental temperature, European ground squirrels show circannual body mass, food and water intake, and, below 15° C, heterothermy/homeothermy rhythms during their entire life span (Andjus et al., 1985, 1988). As reported before (Andjus et al., 2000), under conditions of constant cold (15° and 7° C), in addition to cycles with clearly differentiated phases of activity (148.6 ± 13.8 days in average) and torpor (160 ± 20 days in average) two types of drastically modified rhythms were registered:

1. “accelerated rhythms” (C^A) with unusually high frequency of cycling and extremely short active phases (8–32 days);
2. “protracted hibernation” (C^{PH}) with activity periods restricted to less than seven days;

In this long-term study, we further characterized the effects of moderately low (15° C) and low (7° C) ambient temperatures on body mass and hibernation/activity cycling periods and on the cumulative phase shifting of consecutive cycles.

Materials and Methods

European ground squirrels (*Spermophilus citellus*), born in the laboratory from wild caught pregnant females or trapped in nature, were housed in individual plastic cages and constantly provided with pelleted rodent chow and tap water. They were observed during their entire life span in captivity, ranging from 0.9–8.75 years. After 0.5–1 year at 30° C, one group of animals (eight males and 10 females) remained permanently at 30° C under 12 hour light/12 hour dark regime, while two other groups were transferred to cold rooms at 15° C (five males and five females) or 7° C (six males and six females) in constant dark. They were inspected visually twice daily (at 8 a.m. and 2 p.m.) to record their active state (euthermia) or torpor and the time of death. Weighing of hibernating animals was performed only on days when the animal, following an intermittent arousal, was first found in deep hibernation. Active animals were weighed at seven-day intervals. Body mass (peak-to-peak) oscillation periods (τ_{bm}) were derived from oscillatory body mass profiles constructed using four-point moving averages. The hibernation/activity cycling period (τ_{hib}) was determined as the interval between the ends of two consecutive hibernation bouts. The phase shift of body mass peak or of the end of hibernation was calculated according to the procedure published earlier (Andjus et al., 1985). In brief, the

cumulative shift (its late or early appearance) was expressed in angle degrees in respect to the period of the solar year (365.25 days = 360 degrees). Statistical differences between different periods were calculated using the two-tailed Mann-Whitney test, while the slopes of linear regression lines were compared using t-test. For $P < 0.05$ the differences were considered significant.

Results and Discussion

Retained body mass cycling throughout a life span in captivity under conditions of constant high temperature (30° C) is illustrated in two lower tracings of Fig. 1. Only a few other studies have been performed at such high ambient temperatures (34° or 35° C) and with other species of ground squirrel (*S. lateralis*, Pengelley and Fisher, 1963; Pengelley et al., 1978). In some other studies (*Glis glis*, Mrosovsky, 1977; *Marmota flaviventis*, Ward and Armitage, 1981; *S. richardsonii*, Melnyk, 1983), 20° C ambient temperature was considered as high, although it was far from the temperature of thermal neutrality. Nevertheless, all of them reported body mass cycling in the presence of food and the absence of hibernation. From the recordings in Fig. 1, it is clear that there is a tendency for the reduction of the maximal body weight with aging, demonstrated by comparing the position of the maximum of the body weight cycle with the average body mass (Fig. 1, horizontal line). This reduction of maximal body mass could be the result of lower accumulation of body fat or other physiological changes that accompany the process of aging. In addition to body mass cycling, animals at 15° C and 7° C also exhibited cycles of hibernation/activity periods (Fig. 1).

However, some animals did not show expected cycling behavior. These modified rhythms were more common at 7° C (9 out of 12 animals) than at 15° C (1 out of 10 animals), and C^A were only seen at 7° C. Both animals at 15° C shown in the Fig. 1 had regular cycles. An example of the “accelerated rhythm” is given for the animal at 7° C (Fig. 1; #336). In just 2.82 years at low temperature, this animal completed seven hibernation cycles. The other animal at 7° C (Fig. 1; #526) belongs to the group with “protracted hibernation.” In this case, the active periods were shorter than seven days, during which animal was still able to increase the body mass.

As shown in Table 1, regular rhythms (C^R) were analyzed separately from modified rhythms (C^M). In all cases, τ values were not significantly different between males and females, and they were taken together in calculation. Comparing only regular rhythms, τ_{bm} at 7° C (0.796 ± 0.027 years) was not significantly different from the value obtained at 30° C (0.826 ± 0.013 years).

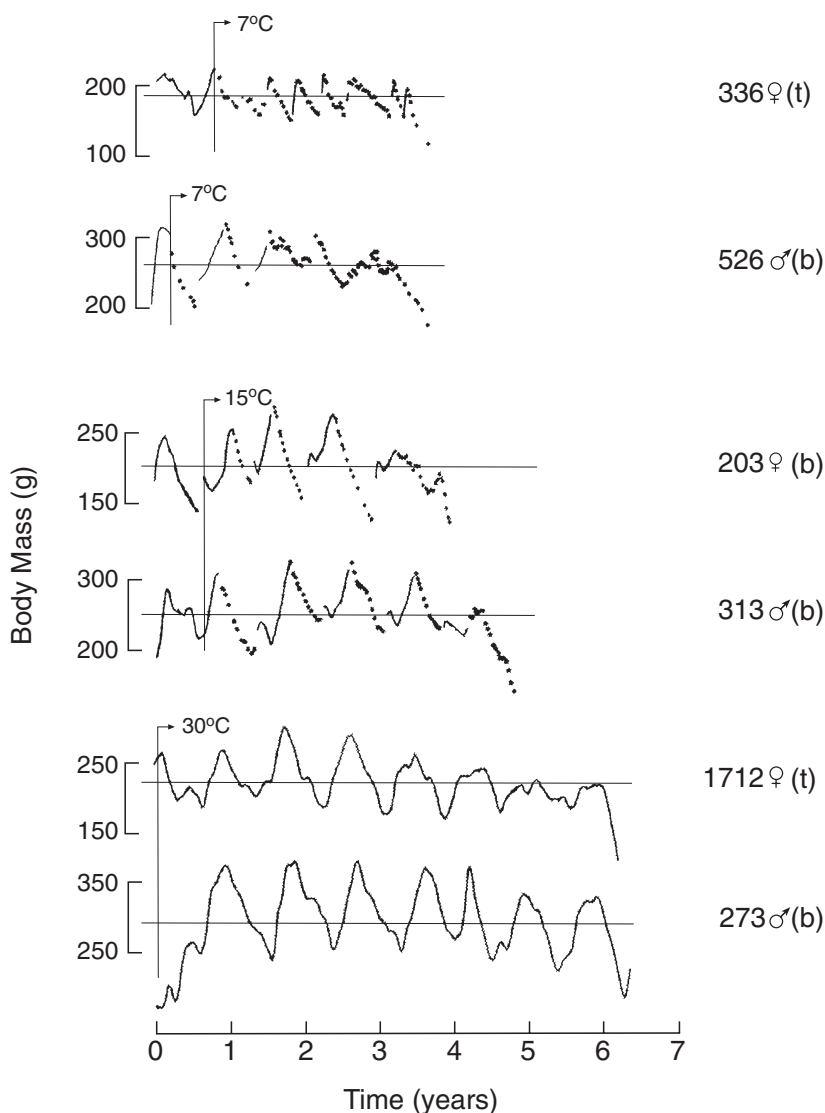


Fig. 1. Examples of circannual cycles of body mass in ground squirrels under free-running conditions at constant temperature (7°, 15°, and 30° C) from birth (b) or the day of capture (t) until death. Animal number and gender are indicated by each panel. Horizontal lines: average body mass; vertical lines: time when animals were transferred to the experimental temperature; full lines: body mass during active phase calculated using four-point moving average; circles: body mass obtained after every second intermittent arousal during hibernation phase.

Table 1. Average free-running body mass (τ_{bm}) and hibernation/activity (τ_{hib}) cycles in ground squirrels maintained at constant temperature.

Temperature regime	life span + SE, in years (range)	τ_{bm} + SE, in years (no. of cycles)	τ_{hib} + SE, in years (no. of cycles)
30° C	6.56 + 0.2 (5.29–8.75)	0.826 + 0.013** (122 cycles)	
	N = 18		
	T: 5.44 + 0.39 (3.73–7.12)		
15° C	C: 4.64 + 0.37 (3.17–6.66)	C ^R : 0.876 + 0.019** (36 cycles)	C ^R : 0.890 + 0.027 (29 cycles)
	N = 10		
	PH: 3.91 (0.90–2.82)	C ^{PH} : 1.107+0.105* (3 cycles)	
7° C	T: 4.04 + 1.21 (2.93–5.63)		
	C: 3.38 + 0.20 (2.44–4.71)	C ^R : 0.796 + 0.027† (23 cycles)	C ^R : 0.840 + 0.032 (14 cycles)
	N = 12		
	M: 2.29 + 0.21 (0.90–2.82)	C ^M : 0.447 + 0.031* (36 cycles)	
	N = 9		

Life span: N = number of animals; T = total life span in captivity (from birth in laboratory-born, from the day of capture in trapped animals); C = life span in constant cold; PH = life span in "protracted hibernation"; M = life span of animals with modified rhythm ("protracted hibernation" or "accelerated rhythm").

τ_{bm} and τ_{hib} : C^R = average of the cycles with the regular rhythms; C^{PH} = average of the cycles only during "protracted hibernation"; C^M = average of the cycles with modified rhythms, "accelerated rhythms," and "protracted hibernation" taken together (* $P < 0.005$ vs. all other τ_{bm} values; † $P < 0.02$ vs. all other τ_{bm} values except those at 30° C, where $P = 0.442$; ** $0.01 < P < 0.05$ vs. all other values marked with the double asterisk).

However, both τ_{bm} 's were statistically shorter than the value obtained for animals at 15° C (0.876 ± 0.019 years). These results are only partially in agreement with the finding of Pengelley and Fisher (1963) that in the temperature range from 0° C to 35° C, the duration of the period is reversibly proportional to the ambient temperature. We have found this relationship only in the range between 30° C and 15° C.

Out of the total number of 181 regular cycles obtained from 40 animals, 13 were longer than a year, but only one of those cycles was recorded at 7° C. Although unusual, Barnes and York (1990) reported for golden-mantled ground squirrel (*S. lateralis*) circannual periods of body mass, reproductive function, and hibernation longer than a year. According to authors, the reason for longer τ 's is not known but could relate to the age or sex of animals (adult males, trapped in spring).

As we expected, for the animals kept at the same temperature, average τ_{bm} values of regular (C^R) and modified cycles (C^{PH} or C^M) were significantly different (Table 1). At 15° C, the τ_{bm} of C^{PH} was longer than a year, but the average τ_{bm} of C^M at 7° C was much shorter than the value for C^R .

We believe that the described modified rhythms are not the consequence of aging, since some animals exhibited the change in the rhythm after the first regular cycle at low temperature. In addition, since the "accelerated rhythms" were registered only at 7° C and not at 15° C, it is possible that this type of modified rhythm was induced by low temperature. "Protracted hibernation" could be the general characteristic of European ground squirrel circannual rhythm under free-running conditions, although we cannot exclude the possibility that other hibernators could exhibit similar behavior. According to Melnyk (1983), *S. richardsonii* displayed six-month cycles at 23° C, but these "fast cycles" were reported recently at low temperature in *S. citellus* (Andjus et al., 2000). On the other hand, there are data in the literature showing acyclic behavior in other species of ground squirrels. Barnes and York (1990) found that 47% of *S. lateralis*, kept at 4° C for two years, permanently interrupted body mass oscillations and stopped hibernating after arising from hibernation and achieving the maximum body weight although they were still exposed to the low temperature. The authors ascribed the loss of cycles in these animals to skipped regression of the gonads after their maturation in the spring, perhaps due to the continuance of low temperatures and lack of warming above a declining threshold of temperature in spring that may be required for the persistence of circannual rhythms ("cold-hold"; Joy and Mrosovsky, 1993). Heller and Poulson (1970) reported extremely

irregular behavior of body weight cycling in *S. beldingi*. During hibernation at 16° C, some animals continued to gain weight and never stopped eating until their death. This phenomenon is to some extent similar to the “protracted hibernation” observed in *S. citellus*, although the latter stopped eating after achieving the maximum body weight and retained its cyclic change.

The difference in τ_{hib} between animals with regular cycles at 15° C and 7° C was not statistically significant ($P = 0.48$), and τ_{hib} and τ_{bm} values of regular cycles at the same temperature were not significantly different ($p = 0.833$ at 15° C; $p = 0.364$ at 7° C) as well. The lack of statistical difference in τ_{hib} at 15° and 7° C can be assigned to a greater variation of data at 7° C.

Since τ 's were shorter than a year and the consecutive cycles appeared earlier and earlier with respect to the first cycle, we wanted to determine the cumulative negative phase shift of body mass and hibernation/activity cycles and assess the temperature dependence (Fig. 2). Slope of regression lines presents the phase shift expressed in degrees/cycle. Number of animals used for calculation of regression lines is given by the symbols, while the total number of cycles taken into account is shown in parenthesis. Slopes of regression lines were compared using t-test. In the case of animals with regular rhythms, the biggest phase shift in body mass cycling was found in animals exposed to 7° C (-80.8 ± 10.9 degrees/cycle, or 81.9 days/cycle; Fig. 2, upper panel) and it is significantly different ($P < 0.001$) from phase shift at 15° (-44.7 ± 6.9 degrees/cycle, or 45.4 days/cycle), but not at 30° C (-66.7 ± 3.0 degrees/cycle, or 67.6 days/cycle). Animals with modified rhythms at 7° C (Fig. 2, upper panel) showed exceptionally big phase shift in body mass cycling (-211.7 ± 5.6 degrees/cycle, equivalent to 211.7 days/cycle), which is 2.6 times the value for animals with regular cycles at the same temperature. Due to short τ_{bm} during modified rhythms (up to 7.2 months shorter than a year), animals could finish several cycles in one year (Fig. 1, animal #336). At 15° C, only one animal showed three cycles with modified rhythm (“protracted hibernation”) and it was not included in Figure 2.

Average τ_{hib} and the corresponding cumulative phase shift for modified cycles were not calculated because it was hard to determine the end of hibernation phase. For regular cycles average phase shifts calculated from regression lines at 15° C and 7° C were not significantly different (Fig. 2, lower panel). Again the absence of statistically significant difference between τ_{hib} at 15° and 7° C was probably due to a greater dispersion of data at 7° C. Comparison of regression lines for body mass and hibernation/activity cycling for regular rhythms at the

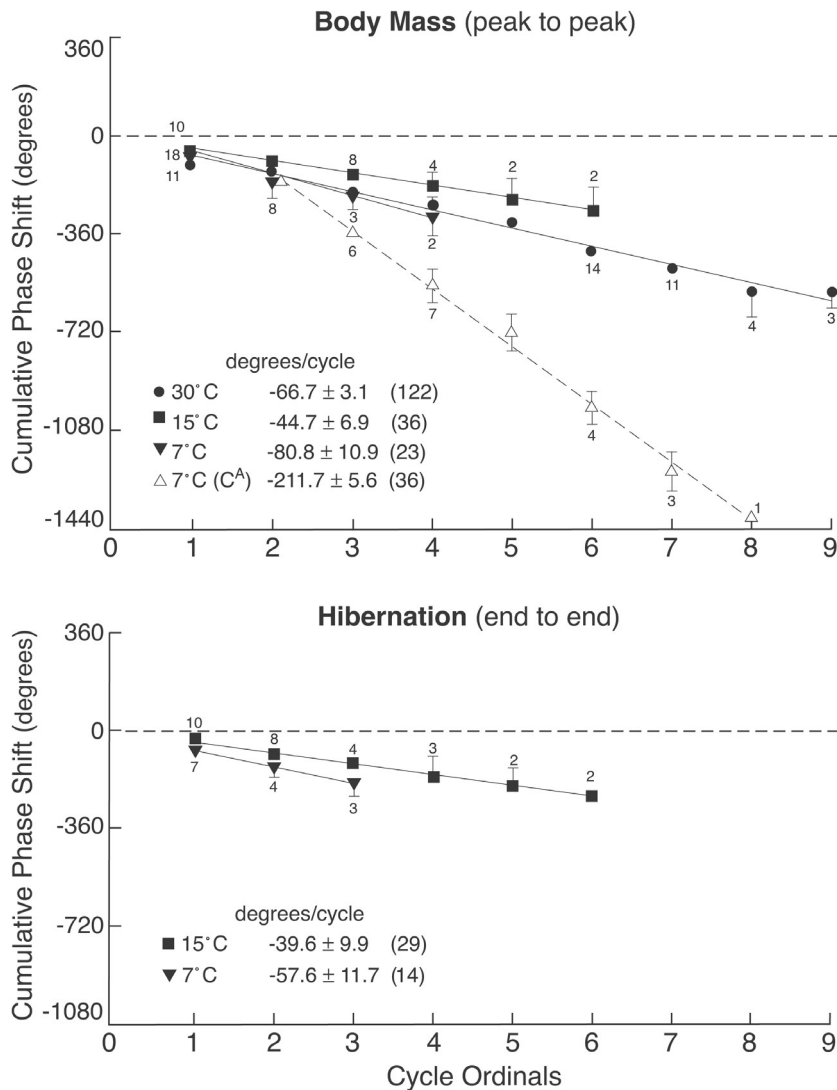


Fig. 2. Average cumulative phase shift of body mass (upper panel) and hibernation/ activity (lower panel) cycles in animals under free-running conditions. Ordinate (degrees): 365.25 days (an average year) = 360 degrees. Regular cycles (filled symbols) and cycles with modified rhythm (open symbols) are presented separately. Numbers adjacent to symbols = number of animals; numbers in parenthesis = number of cycles taken into calculation; inserted tables = slopes of regression lines in degrees/cycle at different temperatures.

same temperature shows that these rhythms are locked. A T-test did not find significant differences between phase shifts at either 7° or 15° C.

Our long-term study suggests that although circannual rhythms in European ground squirrel are temperature dependent, effect of temperature decrease on τ_{bm} cannot be predicted as previously suggested (Pengelley and Fisher, 1963). The results also showed that in free-running conditions besides regular cycles with the period shorter than one year, obtained at 30°, 15°, and 7° C, European ground squirrels (*S. citellus*) displayed modified rhythms ("accelerated" and "protracted hibernation") at a low temperature (7° C). Those rhythms are characterized by extremely short τ_{bm} and particularly big negative cumulative phase shift, resulting in several cycles per year. However, no "accelerated" cycles and only few "protracted hibernation" cycles were recorded at the moderate low temperature (15° C).

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The Role of the Suprachiasmatic Pacemaker (SCN) in Energy Expenditure During Hibernation of Golden-mantled Ground Squirrels

PATRICIA J. DECOURSEY

Department of Biological Sciences, University of South Carolina, Columbia, SC, USA

Abstract. Torpor bouts and brief euthermic arousal episodes alternate in a precise sequence during hibernation of golden-mantled ground squirrels. The characteristics of arousal episodes were quantitatively analyzed for squirrels in natural habitat and also for a group maintained in a simulated natural environment in the laboratory. Wild golden-mantled squirrels, which were completely SCN-lesioned and repatriated in autumn, were not recovered in the field population in spring. The SCN pacemaker apparently fine-tunes the temporal pattern of core body temperature during hibernation and may thereby help in rationing out the vital body fat supply through winter.

Introduction

The suprachiasmatic nucleus (SCN) is the primary circadian pacemaker in mammals and has an important temporal regulatory function in many behavioral and physiological processes (reviews in Weaver, 1998; Takahashi et al., 2001; DeCoursey, 2003). In at least three species of squirrels, the SCN is important for their survival in natural habitats (DeCoursey et al., 2000). SCN involvement in timing of mammalian hibernation has also been shown, at least under laboratory conditions (Ruby et al., 1998).

Many small rodents living in cold climates escape the rigors of winter by fattening before entering a state of long-lasting deep torpor in a deep burrow below the freeze line (Barnes, 1989; Strijkstra, 1999; Ruby, 2003). Through the use of a greatly reduced metabolic rate during the time when food and water

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

are unavailable, the animals can survive autumn drought and winter periods (Körtner and Geiser, 2000). Although the hibernation season may last up to nine months, torpor is interrupted by brief, recurring arousal episodes that usually persist for less than 24 hours (Barnes, 1989; Michener, 1992). The fact that the metabolic cost of arousal episodes is estimated at 86% of total energy expenditure in European ground squirrels (Strijkstra, 1999) implies that precise arousal episode timing is essential in hibernation.

The golden-mantled ground squirrel of the Pacific Cascade Mountains provides an excellent model for examining the function of the SCN pacemaker in energy expenditure during hibernation. The squirrels are highly adapted in their annual cycle to their favored habitat in the high desert ponderosa pine forests. Much is known about SCN regulation of daily activity patterns and about some of the behavioral and physiological events of hibernation in lab-maintained golden-mantled squirrels (Ruby et al., 1998), but little is known for free-living individuals.

The hibernation bout pattern data presented here are based on a study of a population of golden-mantled squirrels from wilderness habitat and from a parallel group maintained in a laboratory hibernaculum under simulated natural conditions. Intact and surgical controls were compared to SCN-lesioned animals.

Material and Methods

In the field study, squirrels were studied for four seasons at a pristine, 20-hectare forest site in the Metolius Research Natural Area, near Camp Sherman, Oregon. In this ideal habitat, population levels of golden-mantled squirrels are high and very stable. Permanent trapping cairns sheltering National live traps were set out in a grid at 50-m intervals in order to gather data from all resident animals at regular intervals during the snow-free season from May to October. Approximately 80 animals inhabited the area in early spring; about 250 were present after emergence of young in early summer. All animals captured on the study site were marked for individual recognition.

A walk-in cold room was used for the hibernaculum to house squirrels in individual cages. The chamber was programmed to track deep ground ambient temperature of the Metolius RNA site on a daily basis. A light schedule was provided, based on daily sunrise/sunset times for Bend, Oregon, with constant darkness from October 15 to March 1.

All project animals selected for intensive study of hibernation patterns were equipped from September 15 through May 15 with a telemetric data logger,

implanted intraperitoneally (Thermochron iButton from Dallas Semiconductor Corp.), which monitored body temperature continuously at three-hour intervals. Extensive efforts were made in the field studies to maximize the retrieval of the data loggers. The protocol consisted of three groups: intact controls, surgical controls (Sham SCN-X), and the SCN-lesioned experimental group. Golden-mantled squirrels for hibernaculum studies were trapped at a site 3 km from the field study site. All animals held in captivity were housed in shoebox-style cages with sufficient food and water to maintain body weight at the level of field animals.

For SCN surgery, animals under deep anesthesia were mounted in a Kopf stereotaxic instrument for alignment of the electrode. The posterior edges of the two orbital zygomatic processes served as a precise skull landmark. Lesion coordinates were determined by constructing a histological atlas for the ventral hypothalamic region by serial histological sectioning of SCN-lesioned calibration animals. A 3-mm diameter skull hole was drilled for insertion of the lesion electrode. Platinum-iridium electrodes from Frederick Haer and Company (FHC) were employed with a current of 25 mA applied for 20 sec.

Results

Field data is based on a total of 30 iButtons recovered from 28 individuals after termination of winter hibernation, plus partial life history data from 54 nonrecovered field animals. The field numbers for retrieved project animals include 18 intact controls, 5 surgical controls and 5 squirrels with partial SCN lesions. The overall recovery rate was 35.7% based on pooled data for all years. The 31 animals used in the hibernaculum included 12 intact controls, two surgical controls, and 17 SCN-lesioned individuals.

Precise, predictable patterns of hibernation bouts and arousal episodes occurred in all field intact control animals. Almost all individuals dropped abruptly from the autumn euthermic level into hibernation, although a few exhibited one or more shallow test drops. Hibernation then proceeded with a regular succession of torpor bouts and euthermic arousal episodes at intervals from 1–15 days. Two distinct sequence patterns are evident. Adult females progressed through a series of hibernation bouts, with body temperature tracking about 2° C above the deep ground temperature. The modulation of the bouts was directly related to ambient deep ground temperature: the colder the ambient temperature, the longer the hibernation bouts (Fig. 1A). The pattern for juveniles of both sexes was similar to adult females (Fig. 1B). A contrasting picture was seen in adult males. Although adult males disappear from the surface in

fall droughts, they often do not enter torpor until late November. Adult males rarely decreased their body temperature below 10° C, which was well above the ambient ground temperature. They usually aroused from hibernation in mid-February when ambient deep ground temperatures were still dropping (Fig. 1C). Their torpor bouts were also quite short.

A consistent pattern for all field controls is clear for the total duration of the hibernation season, the number of arousal bouts, and the average duration of hibernation bouts (Fig. 2). Females and juveniles hibernated for about seven months, with 20–25 torpor bouts averaging 8–10 days and arousal episodes lasting about 24 hours. For breeding males, hibernation generally lasted three months, the number of bouts was less, and the mean duration was shorter.

Only five SCN-lesioned field squirrels were ever recovered in the four years of the study and all were only partially lesioned: three females ranging from 10%–50% SCN remaining, and two males with 20% and 25% SCN remaining. For this reason, the data for SCN effects must come from the hibernaculum data.

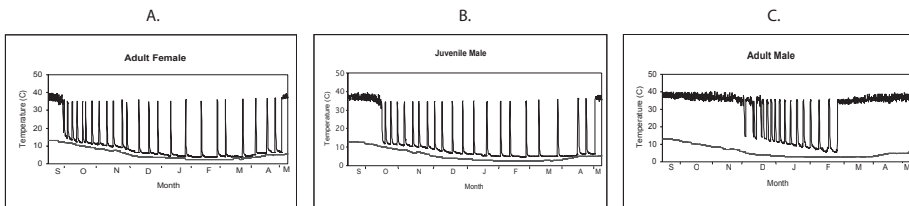


Fig. 1. Representative hibernation pattern of body temperature: (A) adult female; (B) juvenile; and (C) adult male.

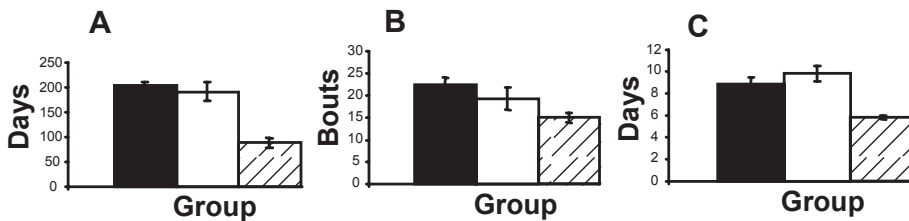


Fig. 2. Parameters of hibernation: (A) total duration; (B) number of bouts; (C) duration of bouts. Symbols: black = adult females; white = juveniles, and hatched = adult males.

Hibernaculum control animals closely resembled field animals in their hibernation duration time, number of bouts, and length of bouts relative to minimal ambient temperature. A major difference between control and SCN-X animals was the presence of aberrant euthermic periods during hibernation (Fig. 3). These abnormal patterns were less pronounced for animals that had undergone SCN-X surgery in early fall than for those lesioned earlier in the year or held more than one year in the hibernaculum. They ranged from very irregular torpor bouts to extended euthermic arousal episodes in midwinter and even a total lack of hibernation.

The relative wakefulness of SCN-X animals potentially heightened the risk of starvation since extra stored fat was required for the extended arousal episodes. After the unexpected death of an early waking intact control male in the hibernaculum, regular feeding was initiated for each individual as soon as it

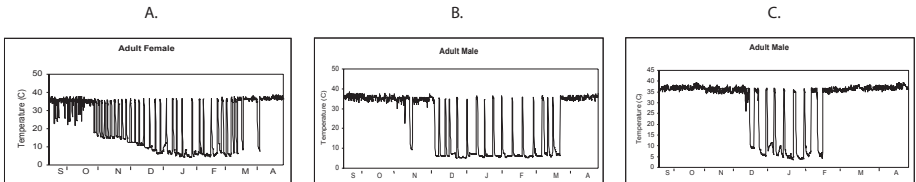


Fig. 3. Hibernation aberrations in SCN-lesioned squirrels. (A) multiple test drops and extended euthermia; (B) irregular arousal bouts; and (C) extended arousal bout.

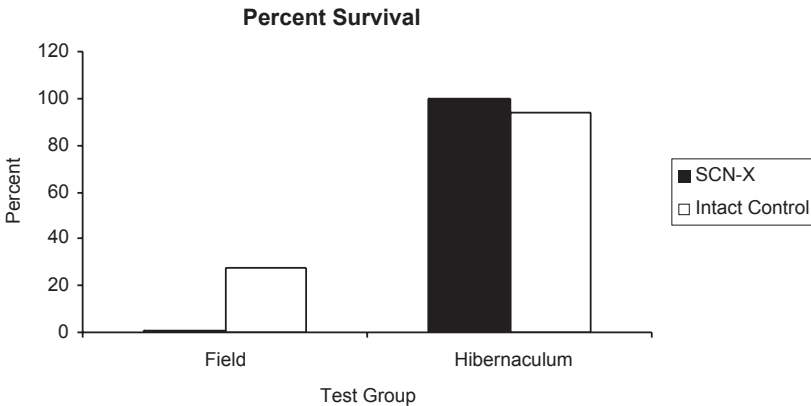


Fig. 4. Survival rate of Intact Controls and SCN-lesioned squirrels at the Field Project site (left) and in the laboratory hibernaculum (right). Symbols: black = SCN-X, white = Intact Control.

terminated hibernation. As long as normal feeding was maintained immediately upon arousal from hibernation, the only difference in survival seen between intact controls and SCN-lesioned squirrels in the hibernaculum was the inadvertent starvation of this one control male (Fig. 4). The difference of survival rate between completely SCN-lesioned animals in the field and in the hibernaculum was one of the most surprising results of the study.

Discussion

Practically no information is currently available concerning the involvement of the SCN pacemaker in hibernation of any wild mammalian species living in natural habitat (DeCoursey, 2000). The lack stems from the extreme difficulty of repatriating SCN-lesioned individuals at their home site after surgery and then assessing their behavioral and physiological performance for the remainder of their lifetime. Telemetric tracking methods such as radio tracking or data logging during the extended period of hibernation are possibilities, but these methods are extremely arduous, time-consuming, and expensive (Michener, 1992; Strijkstra, 1999; DeCoursey et al., 2000). As a result, most data comes from studies of laboratory-reared animals under artificial, nonecological conditions of temperature, light schedule, and feeding.

Laboratory studies have been helpful in characterizing some features of mammalian hibernation, but a comprehensive understanding needs a view of a wild, free-living animal against the backdrop of its natural environment. The holding of golden-mantled squirrels for several years in the laboratory may have conditions that differ greatly from natural field conditions: a constant, relatively high temperature such as 10° C. (Grahn et al., 1994) or 6.5° C, under LD or LL light regimes, with *ad libitum* food supplied (Ruby et al., 1998). For the current study, a pristine site field site free of interference from humans or their domesticated animals was considered an essential design component. Similarly, the hibernaculum was programmed to simulate as closely as possible the annual temperature and lighting cycle of the actual hibernation burrows at the field site.

The two hibernation patterns of intact individuals reflect life history features, particularly energy expenditure and reproductive constraints. In the brief mountain summertime, the squirrels must breed and store enough body fat to last through frequent autumn droughts and bitter winter seasons. Breeding males are photoperiodic and require about eight weeks exposure to increasing day length in the preparatory phase of gonadal growth. Energy source during this extremely cold period is unknown. The iButton field records indicate resumed euthermia

as early as mid-February for adult males, but no sight records of squirrels above ground or evidence for a winter food cache in a male's burrow under field conditions are available. In the present study, some hibernaculum squirrels of both genders stored a small pile of sunflower seeds but most refrained from eating during hibernation. In contrast, adult females arouse from hibernation and mate immediately upon coming to the surface in late April or early May. They seemingly benefit from prolonged hibernation, since a secure surface food supply of spring greens is then available to support them through the subsequent energetically draining gestation and lactation periods. The juveniles can likewise ill afford coming to the surface when a food supply is questionable.

The exact fate of the SCN field project animals is unknown. Extensive trapping failed to relocate any completely lesioned individuals during spring recovery trapping. In contrast, 27.4% of intact animals and surgical controls were retrieved, which is a typical result in most capture-recapture studies. The hibernaculum results give a possible explanation. SCN-lesioning carried out shortly before the start of hibernation had relatively little effect on the body temperature record, and usually caused only slight extensions of the time of euthermia during an arousal episode. In the second year following lesioning, several severe aberrations in body temperature patterns occurred, including continuous euthermia with a total lack of hibernation bouts (unpublished data in progress). As long as a small food reserve was supplied, all SCN-lesioned hibernaculum individuals survived.

Data from body weights of hibernaculum animals throughout hibernation suggest strongly that almost all squirrels are at risk of starvation at the time of arousal from hibernation. The metabolically costly arousal mechanism of even intact controls may bring an individual close to the lethal zone. Any aberrations in euthermia extension during hibernation may tip the balance, particularly for the early-arousing breeding males. Juveniles may enter hibernation with an insufficient fat supply after drought-racked summers and be quite vulnerable to starvation during hibernation. Certainly nonhibernating SCN-X individuals could not survive a winter under field conditions. This fact may help explain the failure to recover any fully lesioned individuals in the field.

In summary, modifications of hibernation pattern and accompanying body weight losses in SCN-deleted animals could result in death by starvation at the critical termination of hibernation in late winter, unless supplementary food is provided. The function of the SCN-pacemaker may be to fine-tune hibernation bout pattern for greatest efficiency in energy expenditure.

Acknowledgements

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Does Hibernation Violate Biological Laws?

ANDRÉ MALAN

Université Louis Pasteur, Neurobiologie des Rythmes, Strasbourg, France

Abstract. Hibernation apparently violates basic principles of allometry and of temperature effects on enzymatic reactions. Since the circadian clock is endowed with similar properties, it might be involved.

Allometry is one of the most universal properties of multicellular living organisms. In particular, resting metabolic rate scales exponentially with body mass, with an exponent of around 0.7 (Heusner, 1991; Darveau et al., 2002; Hochachka et al., 2003). Yet hibernating mammals have long been known to deviate from this rule, with the mass exponent of hibernating metabolic rate, HMR, being close to unity. In other words, mass-specific HMR is mass-independent (Kayser, 1964; Geiser, 1988). This is yet unexplained.

In recent years, another intriguing peculiarity of hibernation has emerged: in certain conditions, HMR is approximately constant over a sizeable range of temperature (Ruf and Heldmaier, 1992; Ortmann and Heldmaier, 2000; Buck and Barnes, 2000). In the present paper, I will show that a similar property may be observed for the duration of hibernation bouts. This observation may provide a clue to the understanding of the other temperature- or mass-independent properties and to the site of their control.

Let us first recall some fundamentals of temperature effects on metabolic reactions. Most of these are catalyzed by enzymes. The reaction rate of an enzymatic reaction is a fraction of its maximum velocity. The size of the fraction depends on the concentration of substrate(s) and if any on that of allosteric effectors. Physiological control affects those concentrations, plus eventually

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the availability of functional enzyme (synthesis and degradation, reversible phosphorylation, etc.).

Even if exothermic, an enzymatic reaction requires a small input of energy to get started, i.e., it has a nonzero energy (enthalpy) of activation. As explained by chemical thermodynamics, this results in a temperature dependency. The van't Hoff-Arrhenius law states that the log of maximal velocity is a linear function of the reciprocal of absolute temperature (in degrees Kelvin), the slope being proportional to the enthalpy of activation (Fig. 1) (Baldwin and Aleksuk, 1973). This is still commonly described as the Q_{10} effect, by reference to an older description of the phenomenon.

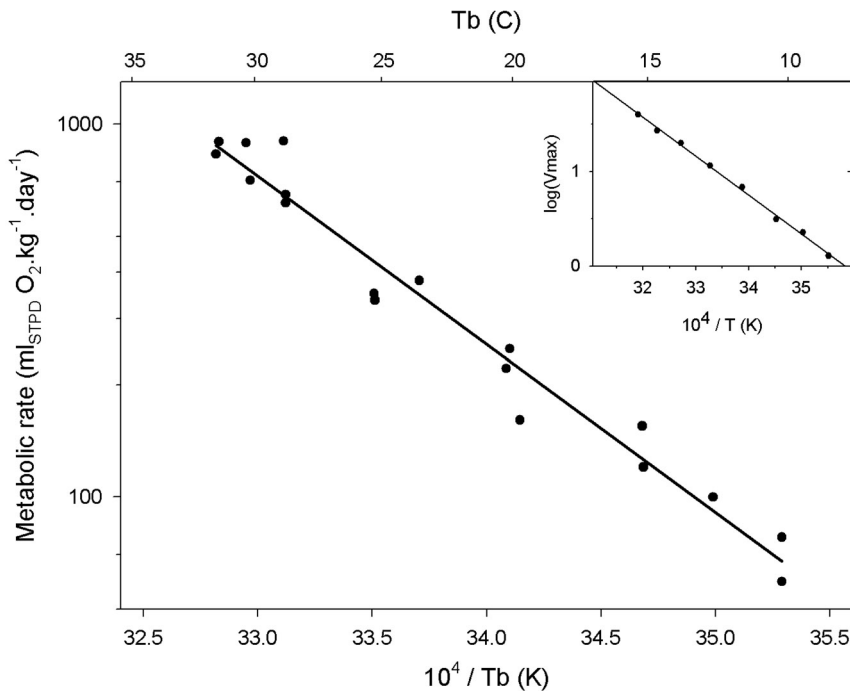


Fig. 1. Inset: As predicted by van't Hoff-Arrhenius law, the logarithm of the maximal velocity of the enzyme LDH (lactic dehydrogenase) from a hibernator is a linear function of the reciprocal of absolute temperature. The slope is proportional to the energy (enthalpy) of activation of the enzyme (data from Baldwin and Aleksuk, 1973). Main panel: The same rate vs. temperature relationship is observed for whole body resting metabolic rate in a crocodile (data from Coulson, 1997).

In the absence of concomitant changes in physiological control of enzymes, the same law of temperature dependency will apply to the overall metabolic rate of the organism, since the whole body oxygen consumption simply is the sum total of those of all mitochondria. The applicability of the vant' Hoff-Arrhenius law to whole organisms has been repeatedly verified (Fig. 1) (Coulson, 1997).

In this context, the occurrence of a regulation of metabolic rate at a temperature-independent level is all the more surprising, since it requires a temperature-dependent correction of the vant' Hoff-Arrhenius effect (Fig. 2). In the environment of the organism, in which all enzymatic reaction rates are affected by temperature (including those involved in the regulation), achieving the temperature independence requires a temperature-independent reference. This is analogous to the problem of a passenger of a moving ship, who wishes to keep an absolute position with reference to the earth by walking towards the

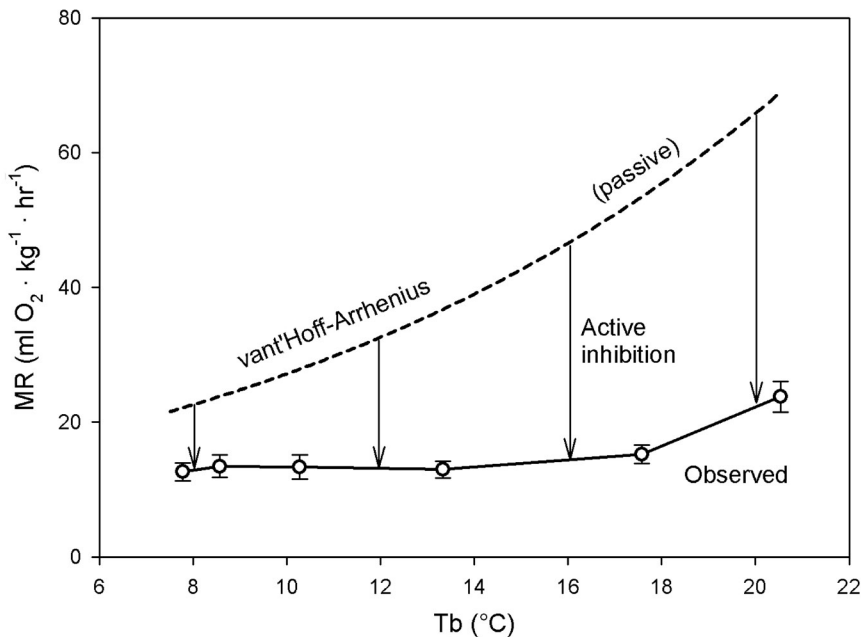


Fig. 2. The metabolic rate of a hibernating arctic ground squirrel is kept constant while body temperature drops from 18° to 8° C (solid line, data from Buck and Barnes, 2000). By comparison, by the passive effect of temperature on reaction rates, metabolic rate would decline with temperature from euthermic resting level following the dashed line. The observed temperature-independent level requires a temperature-dependent active inhibition (arrows).

stern (the speed of the ship is supposed to be low enough to make it possible). Without a GPS or the sight of land, i.e., a reference independent of the motion of the ship, the pedestrian will be totally unable to adjust his speed exactly.

Interestingly, a similar phenomenon of escape from temperature dependence is found in the control of hibernation bout duration, i.e. a period of time, not a metabolic rate. Bout duration is temperature-dependent (Twente et al., 1977; Geiser et al., 1990). Yet a close look at the data of Twente et al. shows that at least in two of the three species of ground squirrel they have extensively studied, the effect of temperature on bout duration is not continuous, but occurs stepwise ($P < 0.01$; Fig. 3). In other words, if only over a few degrees, the control of bout duration can apparently escape the effect of temperature, in the same way as that of metabolic rate. It thus also needs a temperature-independent reference.

The fact that this reference now concerns a period of time gives us a clue. The suprachiasmatic nucleus (SCN) generates endogenous circadian rhythmicity (Reppert and Weaver, 2002). The circadian period is largely, if not totally, temperature-independent (Ruby and Heller, 1996; Ruby, 2003). Could the SCN also provide a temperature-independent reference for hibernation bout duration, irrespective of whether or not it keeps generating an overt circadian rhythmicity during hibernation? The SCN remains metabolically active during hibernation (Kilduff et al., 1990). After SCN lesions, bout duration becomes

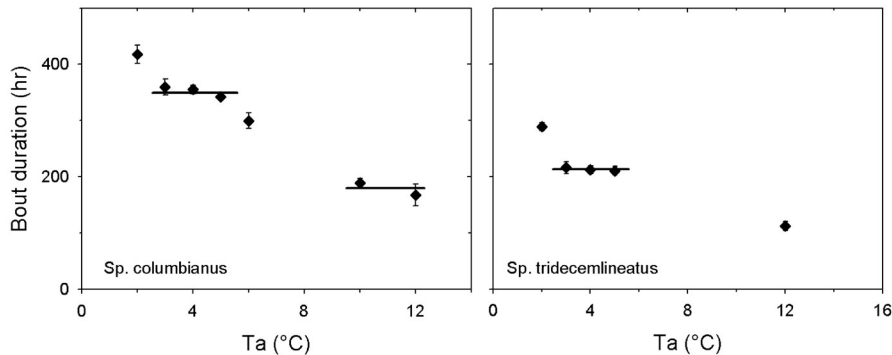


Fig. 3. Hibernation bout duration in *Spermophilus columbianus* (left) and *Sp. tridecemlineatus* (right) vs. temperature (data from Twente et al., 1977). Means \pm SE of pooled animals; slope differences between animals were nonsignificant). Bout duration declined with increasing temperature in a stepwise fashion (comparison with monotonous exponential: $P < 0.01$).

highly erratic (Ruby et al., 1996; Ruby et al., 1998). Melatonin, which modifies the circadian period in the rat (Pitrosky et al., 1999), prolongs bout duration in ground squirrels (Stanton et al., 1987). Conversely, an antagonist of melatonin reduces bout duration in Syrian hamsters (Pitrosky et al., 2003). Although the tau mutation, which reduces the circadian period, does not affect bout duration (Oklejewicz et al., 2001), the SCN is a good candidate as the site of a temperature-independent control of bout duration.

Can the SCN also serve as a temperature-independent reference for hibernation metabolic rate? Here, although the circadian clock is known to be tightly linked with metabolic processes (Rutter et al., 2002), direct experimental evidence is still lacking. We need to know how metabolic rate vs. temperature relationships (Fig. 3) would be affected by SCN lesions.

Another possible link between SCN and hibernation metabolic control is provided by allometry. Allometric relationships are universal. Euthermic metabolic rate scales with body mass with an exponent close to 0.7 (Heusner, 1991; Darveau et al., 2002; Hochachka et al., 2003). This results in the size-dependency of all metabolism-related processes, including many variables that have the dimension of time, such as the period of heart beat. Hibernators are very peculiar in this respect since the allometric exponent of body mass for hibernation metabolic rate is close to unity (Kayser, 1964; Geiser, 1988). In other terms, HMR is size-independent, if bout duration is not (French, 1985).

Among all other metabolism- or time-related physiological variables, only one is known to be size-independent: the endogenous circadian period. It is the same from the shrew to the whale, while heart frequency varies by several orders of magnitude. Here again, SCN may have provided the hibernators with a means to circumvent a fundamental biological constraint.

In conclusion, hibernating mammals have probably made use of mechanisms already present in the circadian clock to achieve a partial temperature-independence of hibernation metabolic rate and of bout duration and to circumvent allometric constraints. This should open the way for future experimental investigations.

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The Suprachiasmatic Nucleus Influences Energy Balance of Golden-mantled Ground Squirrels During Hibernation

NORMAN F. RUBY

Department of Biological Sciences, Stanford University, Stanford, CA 94305

Abstract. This study investigated the role of the suprachiasmatic nucleus (SCN) in hibernation of golden-mantled ground squirrels (*Spermophilus lateralis*) in which the SCN had been completely ablated (SCNx) or left intact. Animals were housed at 6.5° C for 2.5 years. During cold exposure, 50% of SCNx squirrels lost 10–30% of their body mass whereas control animals were unaffected by the cold. Food was available *ad libitum* and several SCNx animals gained body mass during hibernation. Circannual body mass cycles in these animals were replaced by fluctuations in body mass and in duration of individual torpor bouts that appeared to covary. Body mass at the start of a torpor bout and the duration of the subsequent bout were significantly negatively correlated in five of eight SCNx squirrels. The euthermic interval prior to a torpor bout and the subsequent bout were also negatively correlated in seven of eight SCNx squirrels. No significant correlations were found for control animals. These data support a role for the SCN in maintenance of body mass during hibernation and suggest that duration of torpor bouts and euthermic intervals between those bouts can be adjusted to maximize utilization of energy stores.

Introduction

Golden-mantled ground squirrels (*Spermophilus lateralis*) express circannual rhythms in several physiological parameters, including body mass and hibernation (Pengelley and Asmundson, 1974; Zucker et al., 1991). Body mass and hibernation rhythms are temporally coordinated such that the hibernation season begins around the time of year when body mass reaches its peak and ends

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

at the body mass nadir (Fig. 1). Body mass typically decreases linearly over this time while the individual torpor bouts that comprise the hibernation season tend to be shorter at the beginning and end of the season with the longest bouts occurring in the middle of hibernation (Hut et al., 2002; Pengelley and Asmundson, 1974; Ruby et al., 1998). Although these two rhythms maintain a precise temporal relationship across the circannual cycle, the duration of individual torpor bouts is not related to the relative changes in body mass across the hibernation season.

The pattern of shorter torpor bouts at the beginning and end of hibernation with longer bouts in the middle has been reported for many species of hibernators, including golden-mantled ground squirrels. This pattern seems to be consistent among animals studied in the field due to annual changes in burrow temperatures that limit the minimum body temperature (T_b) achieved during torpor (Geiser et al., 1990; Hut et al., 2002; Michener, 1992; Wang, 1978). Torpor bouts tend to be longest in midwinter, in part because burrow temperatures are lowest during that time. Ambient temperature (T_a) cannot, however, completely explain this pattern because a similar annual pattern of torpor bout duration occurs in laboratory conditions where T_a is held constant throughout the hibernation season (Geiser et al., 1990; Ruby et al., 1998), although exceptions have been reported (Dark et al., 1992). The persistence of this pattern of torpor in ground squirrels held without food in constant T_a suggests that seasonal changes in torpor bout duration are either controlled by an endogenous timing mechanism or are driven by other physiological variables that are endogenously timed.

Circannual rhythms in body mass and hibernation, as well as seasonal modulation of torpor bout duration, can be eliminated by ablation of the suprachiasmatic nucleus (SCN). Approximately 50% of ground squirrels in which the SCN was ablated (SCNx) failed to express these rhythms when housed at low temperature ($T_a = 6.5^\circ \text{C}$) even though their body mass rhythms were normal in a warm environment ($T_a = 21^\circ \text{C}$; Ruby et al., 1998). The remaining SCNx animals continued to express body mass rhythms in the cold. Body mass and duration of individual torpor bouts of these animals seemed to fluctuate randomly and SCNx squirrels spent 58% more time torpid than did control animals over the 2.5 years of the study. The mean body mass of individual SCNx squirrels was 10–30% lower in the cold than it was prior to cold exposure. T_a had no effect on the body mass of control animals. The body mass of some SCNx squirrels decreased to life-threatening levels but was restored when they

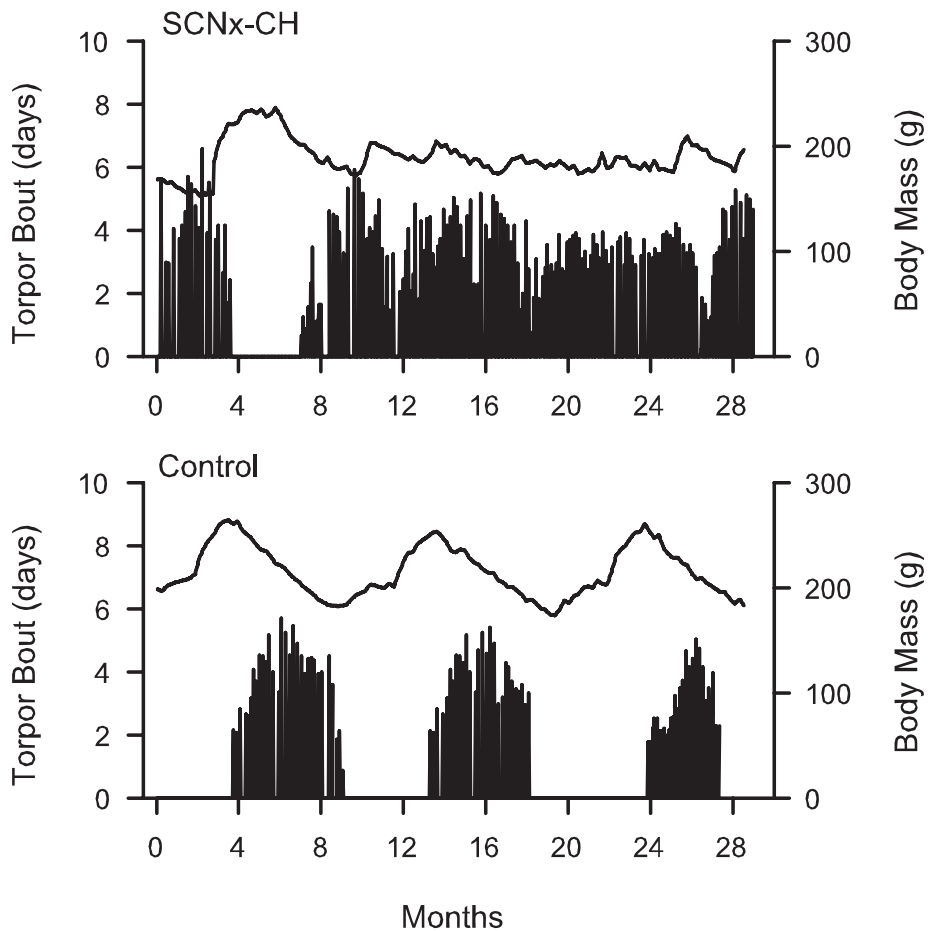


Fig. 1. Representative body mass and hibernation patterns from SCNx-CH and control squirrels. Body mass is indicated by solid black lines and torpor bouts are depicted by vertical bars. Each torpor bout is plotted on the day it was initiated. Note that torpor bouts of the control animals are shortest at the beginning and end of the hibernation seasons. Body mass and hibernation patterns of SCNx-NCH (not shown) were similar to control squirrels.

were temporarily removed from the cold. The loss in body mass occurred even though squirrels were provided an *ad libitum* diet of rodent chow and sunflower seeds, which they ate during the brief euthermic intervals between torpor bouts.

A casual inspection of the data suggested that the dynamic changes in body mass observed among SCNx squirrels appeared to be related to changes in torpor bout duration. This observation prompted a closer analysis of those data to determine whether torpor bout duration was related to changes in body mass in SCNx animals. This was of interest because even though torpor bout duration is not related to relative changes in body mass during hibernation in intact animals, duration of torpor bouts is influenced by the absolute body mass at the onset of the hibernation season (Forger et al., 1986). Thus, changes in torpor bout duration may be due to changes in body mass in SCNx squirrels that lacked circannual rhythms. This hypothesis was tested by correlating body mass at the onset of a torpor bout with the duration of that bout in intact and SCNx animals. Because animals were feeding between torpor bouts, it was also of interest to investigate whether body mass or torpor bout duration was related to the duration of the euthermic intervals between bouts. It was hypothesized that SCNx squirrels spent more time euthermic between bouts because they needed to eat to offset the loss in body mass.

Methods

The methods for all aspects of animal housing, data collection, lesion surgery, and histological analysis of lesion damage for the animals used in this study have been reported in detail (Ruby et al., 1998; Ruby et al., 2002). A brief description of those methods is given here. All squirrels were housed individually at low temperature ($T_a = 6.5^\circ \text{C}$) in a 14:10 light-dark cycle for 19 months and were then housed in constant light for 11 more months.

Lesion Surgery and Histological Verification of Lesion Damage

Bilateral radiofrequency lesions aimed at the SCN were made one to two years before animals were exposed to low temperature when they were at or near their annual body mass minimum. In prior reports (e.g., Ruby et al., 1996), the SCNx squirrels were divided into two distinct groups based on whether they hibernated continuously (SCNx-CH; Fig. 1) or had noncontinuous seasonal hibernation patterns (SCNx-NCH); these designations are retained here. The eight squirrels in the SCNx group sustained complete ablation of the SCN

and had variable damage to adjacent structures. The pattern and extent of that damage did not differ between SCN_x-CH ($n = 4$) and SCN_x-NCH ($n = 4$) animals. Details of neural damage in these animals have been reported (Ruby et al., 1998). Four intact animals served as a control group. Circadian T_b rhythms were eliminated in all SCN_x squirrels (Ruby et al., 2002).

T_b and Body Mass Data Collection

T_b was measured by a biotelemetry system. Female golden-mantled ground squirrels were implanted with temperature-sensitive transmitters and T_b was measured in 10-min intervals over 2.5 years and stored on a computer. Body mass (± 0.1 g) was recorded weekly.

Data Analysis

The circannual rhythms of the animals used in this study were out of phase with one another at the time they were permitted to hibernate in the cold. Therefore, cold exposure began in the middle of the hibernation season for some animals. Likewise, the study was terminated in the middle of hibernation in other squirrels. Therefore, only complete hibernation seasons were used for control and SCN_x-NCH animals; partial seasons were omitted from these analyses. Only torpor bouts >36 h in duration were used so that analyses were done on bouts in which steady-state torpor was achieved.

Body mass values used in the analyses were determined for the day each torpor bout was initiated by linearly interpolating between the weekly body mass values. Thus, Pearson correlations were determined between body mass on the day each torpor bout began and duration of the subsequent bout for each animal. The relationship between body mass and duration of the euthermic interval that occurred before the bout were also evaluated. In addition, the duration of each torpor bout was also correlated with the euthermic interval that preceded it. Correlations were considered significant if $P < 0.05$.

Results

Detailed analysis of body mass and hibernation patterns for the animals used in this study have been published elsewhere (Ruby et al., 1998). Representative body mass and hibernation patterns for SCN_x-CH and control animals are shown here (Fig. 1). There was a significant negative correlation between body mass at the start of a torpor bout and subsequent duration of that bout for five of eight SCN_x animals (three SCN_x-CH, two SCN_x-NCH) and for one of four

control animals (Fig. 2). This one control animal had an unusually short hibernation season and terminated hibernation after the longest bout of the season. Among SCNx squirrels, there was a tendency for torpor bouts to increase in duration when body mass was low (Fig. 2).

There were no significant correlations between body mass and euthermic intervals for any of the animals in the study (data not shown). There were, however, significant negative correlations between the duration of a torpor bout and the euthermic interval that preceded it for seven of eight SCNx squirrels (four SCNx-CH and three SCNx-NCH) but not for any of the control animals (Fig. 3). Among SCNx animals, euthermic intervals between torpor bouts tended to shorten as torpor bouts increased in duration.

Discussion

A role for the SCN in maintenance of energy balance during hibernation was previously proposed (Ruby et al., 1998; Ruby, 2003). That proposition was based on the exaggerated body mass loss experienced by SCNx squirrels that were exposed to low T_a s. Those animals lost 10–30% more mass than did control animals over a 2.5-year period and this effect was greatest among SCNx-CH squirrels (Ruby et al., 1998). Food was continuously available to these animals and the body mass increases during hibernation show that they were eating during the euthermic intervals between bouts of torpor. Although food was also available to control animals, it is unclear whether they ate when aroused because they did not exhibit body mass increases during hibernation. Nevertheless, SCNx animals were not able to consume enough food to offset their body mass loss. SCNx squirrels aroused much more frequently during hibernation than did control animals (Ruby et al., 1996). The primary energy expenditure during hibernation is the periodic arousals from torpor (Wang, 1978), thus, the exaggerated body mass loss experienced by SCNx animals is likely due to increased energy expenditure associated with their frequent arousals. Alternatively, SCN ablation may have disrupted energy storage mechanisms or the internal assessment of available metabolic fuels.

In the absence of the SCN, torpor bout duration increased significantly when body mass approached low values. This trend was most pronounced among SCNx-CH squirrels, which lacked any seasonality in their hibernation patterns. Given that the changes in body mass during hibernation are mainly due to changes in lipid mass (Dark et al., 1989), mechanisms that control torpor bout duration may also monitor available energy reserves. One study reduced body

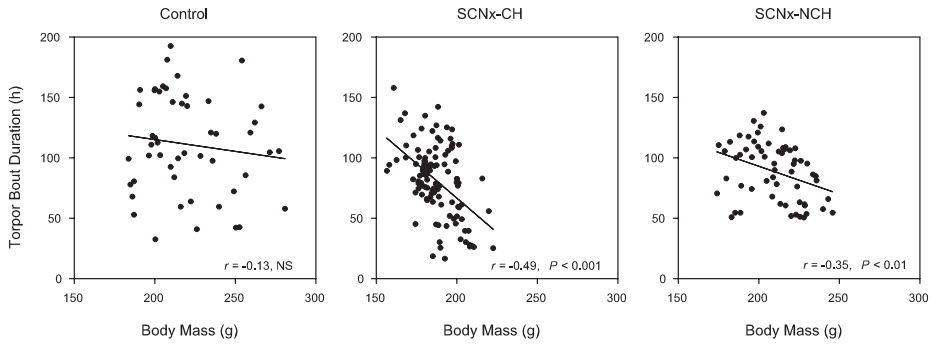


Fig. 2. Representative scatter plots for body mass and torpor bout duration for three individual control, SCNx-CH, and SCNx-NCH squirrels. Correlation coefficients are given for each plot. NS, nonsignificant ($P > 0.05$).

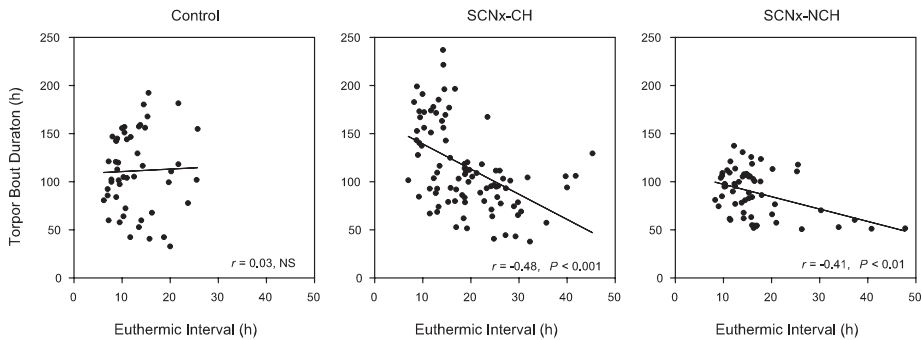


Fig. 3. Representative scatter plots for torpor bout duration and euthermic intervals for three individual control, SCNx-CH, and SCNx-NCH squirrels. Correlation coefficients are given for each plot. NS, nonsignificant ($P > 0.05$).

mass in golden-mantled ground squirrels prior to the hibernation season by either restricting their food intake or surgically removing fat depots (Forger et al., 1986). Animals in both groups hibernated normally but had significantly longer torpor bouts compared to intact control animals. Conversely, supplementing food stores of free-ranging chipmunks, and thereby increasing their lipid mass, decreased the amount of time spent in torpor (Humphries et al., 2003). Low body mass among SCNx squirrels in the present study was likely due to low lipid stores. Decreases in fat mass may signal the need to increase torpor bout

duration to conserve fuel stores. The relationship between body mass and torpor bout duration is not normally seen in intact animals because of their steady linear decrease in body mass during hibernation. Ablation of the SCN altered the relationship between body mass and torpor in a manner that suggests the SCN is somehow involved in energy balance during hibernation.

In addition to increasing torpor bout duration, energy stores could also be preserved by minimizing the amount of time an animal is euthermic. This was the case for SCN_x squirrels, which markedly decreased the duration of their euthermic intervals between bouts as torpor bouts increased in duration. There was not, however, any direct relation between body mass and euthermic intervals in any of the SCN_x animals. It is noteworthy that body mass was unaffected by SCN ablation as long as animals were housed in a warm room, but was only compromised when animals were housed in the cold, an effect not experienced by control animals. It is compelling to suggest that SCN ablation compromises the squirrel's ability to defend their body mass in the cold and that, in turn, the duration of torpor bouts and euthermic intervals was adjusted to preserve available energy stores. If this were true, however, one would expect SCN_x animals to arouse less frequently, but in fact they arouse much more often than control animals, suggesting that there is a limit to how much a torpor bout can be extended. The relationships among body mass, torpor bout duration, and euthermic intervals are likely much more complex than can be discerned by simple correlation analysis and will require more sophisticated statistical models to elucidate causal relationships among these variables.

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Pesticide Effects on Body Temperature of Torpid/Hibernating Rodents (*Peromyscus leucopus* and *Spermophilus tridecemlineatus*)

THOMAS E. TOMASI,¹ PETA ELSKEN-LACY,¹ JEAN A. PERRY,¹ AND KERRY WITHERS²

¹ Department of Biology, Southwest Missouri State University, Springfield, Missouri, USA

² Department of Biological and Physical Sciences, University of Southern Queensland, Toowoomba, Queensland, Australia

Abstract. Environmental contaminants have been shown in the lab to alter thyroid hormone concentrations. Despite the role these hormones play in the physiological ecology of small mammals, no one has investigated the possible effects of thyroid-disrupting chemicals on mammalian thermal ecology and thermoregulatory ability. Because the energetic impact of such a disruption is likely to be most dramatic during times already energetically stressful, we investigated the effects of two common pesticides (atrazine and lindane) on the use of daily torpor in white-footed mice, and the use of hibernation in thirteen-lined ground squirrels. Fortunately, we found that these strategies for over-wintering success were not impaired.

Introduction

Recent studies have provided convincing evidence that a number of chemicals have disruptive effects on the endocrine system (Colborn and Clement, 1992). These “endocrine disruptors” include many pesticides and polychlorinated biphenyls (PCBs) and can compromise an animal’s survival, or a species’ continued existence, without directly killing any individuals. Because winter is already an energetically stressful time of year, any additional stress imposed by an endocrine disruptor may be especially detrimental at this time.

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For mammals, the energetic difficulties associated with winter are two-fold. First, since reduction of heat loss is not usually sufficient, there are the increased thermoregulatory demands of remaining euthermic. These must be met via either shivering or nonshivering thermogenesis (NST). Second, winter is usually associated with decreased quantity and quality of available food. To avoid these energetic problems, many species of small mammals have evolved the ability to enter torpor, either on a limited basis (daily torpor) or a seasonal basis (hibernation).

The relationship between mammalian thermoregulation, torpor, and the thyroid gland has been examined extensively but is still not completely understood. We know that thyroid function in small mammals is altered by cold exposure and by approaching winter (Tomasi and Mitchell, 1996), and that thyroid changes occur prior to and during hibernation (Tomasi and Stribling, 1996; Tomasi et al., 1998; Nicol et al., 2000). Since brown fat function is also dependent upon thyroid hormones (thyroxine – T_4 and triiodothyronine – T_3), changes in the plasma levels could affect thermogenesis and/or the use of daily torpor (Himms-Hagen, 1990). Because of these relationships, improper thyroid function could potentially interfere with the use of torpor.

Many environmental contaminants have been documented to alter thyroid function, mostly in laboratory rodents (Brucker-Davis, 1998; Crisp et al., 1998), either inhibiting the thyroidal system or mimicking it (Cheek et al., 1999). Possible mechanisms for this disruption include direct interaction with thyroid hormone receptors in target cells, either as agonists or antagonists; altering serum transport of thyroid hormones; or interfering with thyroid hormone and cell membrane interactions. In most of these cases, exposure to such compounds is correlated with decreased serum levels of thyroid hormone, particularly thyroxine (Colborn et al., 1996; Curran and DeGroot, 1991), and it is assumed that there may be some negative consequences to this disruption of normal thyroid function. Unfortunately, direct measurements of parameters linked to energetics and winter survival are rare (French et al., 2001; Tomasi et al., 2001).

The emerging evidence linking synthetic chemicals to thyroid disruption is especially worrisome because of the potential bioaccumulation and biomagnification of these chemical within animals feeding at different levels of the food chain (Guillette et al., 1995). Some are extremely persistent in the environment, taking decades to break down. Lipophilic contaminants are also stored in animals' fat reserves until they are metabolized during energetically expensive events, such as thermogenesis in the cold. Furthermore, since hibernating mammals accumu-

late extra body fat during the prehibernation period, and “live” off this fat during hibernation, any lipophilic toxins will be released from the fat all winter.

Atrazine is a triazine herbicide and is reported to be the most widely used herbicide in the world (Hayes et al., 2002). It is used for weed control in agriculture and is highly persistent in soil and water. Atrazine effects on thyroid function (reviewed by Brucker-Davis, 1998) include increased plasma levels of T_4 and T_3 .

Lindane is an organochlorine, and the active component of the insecticide hexachlorocyclohexane (HCH). It is used on agricultural crops, and in the treatment of hardwood logs and stored grain products. It is also highly persistent in soil and water, and its effects on thyroid function (reviewed by Brucker-Davis, 1998) include reduced plasma levels of T_4 and T_3 and elevated thyroid-stimulating hormone (TSH).

White-footed mice (*Peromyscus leucopus*) use daily torpor when the energetic conditions dictate. These torpor bouts in white-footed mice and in deer mice (*P. maniculatus*) usually last from 2–10 hours. Body temperature (T_b) is typically reduced from about 37° C to around 20° C, while metabolic rate is reduced by 75% (Gaertner et al., 1973; Nestler, 1991; Tannenbaum and Pivorun, 1988).

The thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) is widely distributed in the northern Great Plains of the USA. It readily uses deep seasonal torpor (hibernation) generally from October to March, depending on latitude, age, and gender (Streubal and Fitzgerald, 1978).

Although previous studies suggest that pesticides can alter thyroid function by interfering with thyroid hormone levels in laboratory rodents, it is also important to determine if such a disruption will ultimately affect over-winter survival of rodents under natural conditions (Van den Berg, et al. 1991; Brucker-Davis, 1998).

The objectives of this study are to determine whether the purported thyroid-disrupting chemicals atrazine and lindane disrupt thyroid function such that they interfere with temperature regulation during euthermia, during bouts of daily torpor in white-footed mice, and during hibernation in thirteen-lined ground squirrels. Rates of metabolism and concentrations of thyroid hormones were also measured but are not reported herein.

Methods

White-footed mice ($n = 18$) were trapped (Sherman traps) in southwest Missouri (Greene County), and thirteen-lined ground squirrels ($n = 30$) were trapped (tube traps) in northwest Iowa (Dickinson County). Both were housed in an

approved animal room in the Biology Department of Southwest Missouri State University, in individual polypropylene cages (16x20x27 cm) maintained in environmental chambers under controlled temperature and photoperiod. Both protocols were approved by the SMSU IACUC.

Animals were provided *ad lib.* rodent diet (Purina #2010) and water, except when food was reduced for white-footed mice to 75% of *ad lib.* consumption to induce torpor. To simulate seasonal changes for these mice, the photoperiod was gradually reduced from 13L:11D to 9L:15D, and the ambient temperature (T_a) was reduced gradually from 18/13° C (day/night) to 13/8° C. For the ground squirrels, hibernation was induced by a gradual reduction of the photoperiod from 14L:10D to 8L:16D, followed by a reduction of T_a from 26° C to 5° C.

Pesticide (herbicide or insecticide) treatments were prepared by spraying each pesticide solution onto a single layer of rodent chow, and letting this chow then dry before feeding it to the animals. "Control" chow was sprayed with water.

For the white-footed mice, four treatments groups were established (three atrazine doses and a control, with males and females divided evenly among treatments), and an application rate of 25 mL/2.5 kg of chow was used. The atrazine treatments, based on an average food consumption of approximately 4 g/day, were: "low" = 0.376 ug/day (0.235 mg/25mL, 0.094 mg/kg chow); "medium" = 0.075 mg/day (47 mg/25mL, 18.8 mg/kg chow); and "high" = 1.5 mg/day (940 mg/25mL, 376 mg/kg chow). The low dose was chosen to match the recommended crop application rate and the higher doses to simulate possible bioaccumulation in the environment. Each dose was administered chronically, starting in mid-September. The food restriction to induce torpor was started 15 weeks later, three weeks prior to the onset of T_b data collection, and continued for about six more weeks.

For the thirteen-lined ground squirrels, five treatment groups were established (four atrazine and/or lindane doses, plus a control), each with comparable males/females, and 1.96 kg of food for each group was sprayed with 280 ml of the appropriate solution. Treatments were based on a mean food consumption of 35 g/day, and consisted of: "high lindane" = 17.5 mg/day (0.98 g/280mL, 500 mg/kg chow); "low lindane" = 1.75 mg/day (0.098g lindane/280mL, 50 mg/kg chow); "atrazine" = 205 mg/day (11.508 g/280mL, 5871 mg/kg chow); and "low lindane/atrazine." The latter treatment received both solutions applied to its food, and was designed to determine if any synergistic effect occurred. Treatments were started six weeks before measurements were initiated, and were maintained for the duration of the study (six months).

Body temperatures of white-footed mice (measured eight at a time) were continuously monitored (every three minutes) for six to eight days at 75% of *ad lib.* food intake, using implanted (IP, under sodium pentobarbitol anesthesia) temperature-sensitive transmitters and automated data acquisition system (VM-FH transmitters and Vital View Series 3000: Mini-Mitter Co., Sun River, OR). Torpor was defined as T_b below 30° C. Each group of mice monitored simultaneously included individuals from all treatments. Data for each torpor bout were analyzed for mean euthermic (midnight–4:00 a.m.) and minimum temperatures (° C), time spent in torpor, torpor “area” (a calculated integration of torpor depth and duration: degree-hours), and heat loss and heat gain rates between 25° C and 30° C (° C/hr). Parameters for an animal were calculated for each bout with reliable T_b recordings, and data from multiple torpor bouts were averaged for that animal to avoid pseudoreplication.

Body temperatures of thirteen-lined ground squirrels (measured 12 at a time) were continuously (every 10 minutes) monitored for approximately four weeks with VM-XF-LT-DISC transmitters and the hardware/software described above. Each group of ground squirrels monitored simultaneously included individuals from all treatments. Data for each hibernation bout were analyzed for euthermic and torpid mean temperatures (° C), and heat loss and heat gain rates (° C/hr) between 10° C and 30° C. Because T_b data obtained while entering and arousing from torpor were used to calculate heat loss and gain rates, we defined “torpor duration” as time spent under 10° C. For animals that maintained a hibernating T_b at or near 10° C, the heat loss rate was calculated 30° C to 15° C, and the bout was considered terminated when the animal initiated a rapid increase in T_b . Data from multiple hibernation bouts were averaged to avoid pseudoreplication. Shallow bouts at the beginning of the hibernation season were excluded.

The data were analyzed via one-way ANOVA (Minitab), using a GLM procedure and $P \leq 0.05$ for significance.

Results

Most of the white-footed mice entered torpor fairly regularly with a 25% reduction in food. Their euthermic temperatures were stable at 36–37° C, and their torpor bouts generally lasted 3–7 h with a minimum temperature about 20° C (Table 1). When entering torpor, they cooled down about 50% as quickly as they heated up when arousing from torpor. Although the mice that received the low dose of atrazine appeared to use torpor less, with a torpor “area” of less than half that seen in the other treatment groups, this was not statistically significant

($P = 0.07$). The mice showed no atrazine-treatment effects on any of the torpor parameters measured (Table 1).

The thirteen-lined ground squirrels showed typical hibernation/arousal patterns, arousing about every four to seven days (longer in the middle of the hibernation season). Including the torpor entry and arousal times in each torpor bout increases the torpor duration by about 8 hours but demonstrates the same statistical results. Animals maintained T_b a few degrees above the environmental chamber (5°C) while torpid (Table 2). Their rate of heat gain during an arousal was similar to that of the white-footed mice, but their rate of heat loss when entering torpor was only about 12%–22% of this rate. However, none of these parameters varied among pesticide treatments (Table 2).

Discussion

Under our simulated laboratory conditions, both species used torpor in predictable manners, based on previous studies on these or related species (Stribling and Tomasi, unpubl data; Barnes and Ritter, 1993; Gaertner et al., 1973; Nestler, 1991; Tannenbaum and Pivorun, 1988). Both species also demonstrated a typi-

Table 1. Body temperature (T_b) parameters in relation to daily torpor bouts, in white-footed mice ($n = 18$) chronically fed atrazine-treated food (means \pm SE).

Parameters n =	Atrazine Treatment				P =
	Control 3–6	Low 4	Medium 4–5	High 4	
Torpor frequency (% of days)	87 \pm 13	70 \pm 10	78 \pm 20	89 \pm 7	0.79
Euthermic T_b ($^\circ\text{C}$)	36.36 \pm 0.23	36.95 \pm 0.29	36.70 \pm 0.30	36.33 \pm 0.25	0.37
Torpor T_b ($^\circ\text{C}$)	20.54 \pm 1.39	22.93 \pm 1.49	19.15 \pm 0.57	19.38 \pm 0.13	0.13
Torpor duration (h)	5.42 \pm 1.18	3.26 \pm 0.62	6.31 \pm 0.60	6.05 \pm 0.38	0.10
Heat loss rate ($^\circ\text{C}/\text{h}$) [30–25 $^\circ\text{C}$]	8.20 \pm 0.86	7.05 \pm 0.53	8.90 \pm 0.50	8.80 \pm 0.54	0.25
Heat gain rate ($^\circ\text{C}/\text{h}$) [25–30 $^\circ\text{C}$]	14.30 \pm 1.94	17.60 \pm 4.40	20.85 \pm 1.88	18.93 \pm 4.06	0.51
Torpor “area” (degree-hours)	48.32 \pm 11.74	21.03 \pm 6.48	57.17 \pm 9.86	50.24 \pm 2.68	0.07

Table 2. Body temperature (T_b) parameters in relation to hibernation bouts in thirteen-lined ground squirrels ($n = 30$) chronically fed atrazine-treated or lindane-treated (high or low dose) food (means \pm SE).

Parameters	Pesticide Treatment					P =
	Control	Atrazine	Atrazine/ lindane (L)	Lindane (L)	Lindane (H)	
n =	5	5–6	6	6	6–7	
Euthermic T_b ($^{\circ}$ C)	36.39 \pm 0.26	36.23 \pm 0.15	36.23 \pm 0.26	35.81 \pm 0.16	36.14 \pm 0.18	0.38
Hibernation T_b ($^{\circ}$ C)	6.49 \pm 0.38	7.93 \pm 0.71	8.36 \pm 0.75	7.70 \pm 0.32	7.60 \pm 0.70	0.35
Hibernation bout duration (h)	78.8 \pm 19.8	139.9 \pm 32.4	157.8 \pm 20.7	121.4 \pm 18.3	168.6 \pm 30.0	0.16
Heat loss rate ($^{\circ}$ C/h) [30–10 $^{\circ}$ C]	3.08 \pm 0.25	3.64 \pm 0.76	3.32 \pm 0.40	2.42 \pm 0.37	2.58 \pm 0.14	0.50
Heat gain rate ($^{\circ}$ C/h) [10–30 $^{\circ}$ C]	20.02 \pm 1.60	16.65 \pm 2.19	18.30 \pm 1.70	16.17 \pm 1.47	21.15 \pm 1.53	0.20

cal eutherian T_b . The slower cooling rate of the ground squirrels is likely related to their larger body mass (allowing more insulation) and lower surface-to-volume ratio, so it is interesting that they were able to produce heat fast enough to warm up at the same rate as the mice. This might be explained by the same higher insulation and lower surface-to-volume ratio that resists passive cooling, if this species also has more NST capacity, a logical prediction for an obligate hibernator *vs.* a facultative daily-torpor species.

It appears that the pesticides used in this study (at these doses) do not impact the use of torpor in these species, although they have been reported to cause thyroid disruption (Brucker-Davis, 1998; Crisp et al., 1998). This lack of significant physiological effects may be due to small sample sizes; however, the equipment hardware limited the number of transmitters that can be monitored simultaneously. As we rotated different groups of animals onto the receivers, the groups contained individuals from each pesticide treatment, to minimize seasonal effects, but any such effect would still increase the within-treatment variation. In addition, to avoid pseudoreplication, we further reduced our sample size by using in

this analysis the average value for each animal. When statistically separating the within-individual variation from the treatment variation (ANOVA with “animal” nested within “treatment”), and using all torpor/hibernation bouts for which we have reliable data, some of the P-values are reduced below 0.05. Finally, using our operational definitions for the duration of torpor in mice ($T_b < 30^\circ \text{C}$) and ground squirrels ($T_b < 10^\circ \text{C}$), and the T_b ranges for calculating rates of temperature change ($25\text{--}30^\circ \text{C}$ and $10\text{--}30^\circ \text{C}$, respectively) was somewhat arbitrary. These were chosen to avoid inclusion of nontorpor variations in T_b and to obtain values for maximum rates of T_b change. Recalculations using different temperature ranges generally lead to the same statistical conclusions.

The animals used in this study were also subjected to measurements of resting metabolism (nontorpid), NST capacity, and serum concentrations of thyroid hormones (Elsken-Lacy, unpublished thesis data; Perry, unpublished thesis data). In general, the results from those studies support the conclusions of the T_b data presented here. There were few parameters where significant differences were obtained between treatment groups, and these were not sufficiently consistent to conclude that exposure to these levels of these pesticide would negatively impact the over-wintering success of these species in the wild.

The lack of energetic problems noted in these studies, despite previous reports that these pesticides are thyroid disruptors at similar doses (Brucker-Davis, 1998, Crisp et al., 1998), is good news for the wild rodents and for the people who manufacture and use these pesticides. However, we believe that guarded optimism is appropriate. The potential still exists that man-made thyroid-disrupting chemicals are affecting the delicate energetic balance of nontarget species in subtle ways, compromising their over-wintering ability or reducing the reproductive potential of survivors in the following year. Vigilance is still necessary, and thyroid-disruption possibilities should be investigated where poor over-wintering success is otherwise unexplained.

There are many possible explanations for the different conclusions between previous studies on lab rodents and the studies reported here. Since previous studies only measured thyroid hormone levels, and ours are among the first to include ecologically relevant energetic parameters, it is possible that the laboratory rodents were not really compromised. They may compensate for decreased serum levels by altering some other aspect of thyroid function or metabolism not monitored in those studies. Alternatively, wild rodents (particularly the herbivorous species) may possess a better ability to detoxify pesticides because of their continuous exposure to the numerous toxic plant secondary compounds in their diets.

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Steroidogenesis and the HPA Axis During Hibernation: Differential Expression of the StAR Protein

MATTHEW T. ANDREWS, MEAGHAN M. TREDREA, AND AUBIE K. SHAW
University of Minnesota Duluth, Department of Biology, Duluth, Minnesota, USA

Abstract. This chapter examines a rate-limiting component of the hypothalamic-pituitary-adrenal (HPA) axis during mammalian hibernation. Specifically, we have analyzed the seasonal expression of the steroidogenic acute regulatory protein (StAR) in the adrenals of the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*. StAR activity is a rate-limiting step in steroidogenesis and is required for production of a class of steroid hormones that regulate metabolism called glucocorticoids. In ground squirrels, physiologically important glucocorticoids include cortisol, and to a lesser extent, corticosterone. We have hypothesized that regulation of adrenal steroidogenesis during hibernation is mediated by changes in the concentration of StAR protein. We found that levels of StAR mRNA and protein decline during the autumn months and are significantly reduced during deep hibernation when animals show depressed metabolism and body temperatures of 4–6° C. These results suggest that the StAR protein may play an important role in regulating the hibernation phenotype.

Introduction

Time of year, shorter day length, low ambient temperatures, and reduced food availability are thought to induce neuroendocrine signals that are relayed to various tissues throughout the body in order to initiate the hibernation phenotype (reviewed in Carey et al., 2003). This chapter examines the role of one of the major neuroendocrine systems during mammalian hibernation, the hypothalamic-pituitary-adrenal (HPA) axis. Specifically, we have analyzed a rate-limiting step in steroidogenesis in the adrenal cortex of the thirteen-lined

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ground squirrel, *Spermophilus tridecemlineatus*. The adrenal cortex secretes two types of steroid hormones: glucocorticoids and mineralocorticoids. A major glucocorticoid is cortisol and the main mineralocorticoid is aldosterone (Goodman, 1994). As their names imply, the primary activity of glucocorticoids is the regulation of glucose metabolism, while the primary activity of mineralocorticoids is regulation of mineral (e.g. sodium) and fluid balance.

The HPA axis likely plays an important role in triggering the physiological changes that allow animals to hibernate, as evidenced by previous studies where removal of the adrenals disrupts hibernation (Popovic et al., 1957; Vidovic and Popovic, 1954). The basic mechanism of the HPA axis in regulating glucocorticoid production involves environmental influences that induce the hypothalamus to secrete corticotropin-releasing hormone (CRH), which signals the pituitary. The pituitary responds by secreting adrenocorticotrophic hormone (ACTH) into the bloodstream where it acts on the adrenal cortex to stimulate secretion of cortisol. Secretion of cortisol requires (1) *de novo* synthesis of steroid precursors from cholesterol that is transported into the mitochondrial matrix, (2) modification of steroid precursors in microsomes, and (3) packaging of cortisol into secretory granules. Although cortisol has a number of dramatic effects on physiology, how it relates to hibernation is still unknown.

One of the first steps of steroidogenesis within the adrenal cortex is the transport of cholesterol from the outer to the inner mitochondrial membrane. Once cholesterol has reached the inner membrane, it can be cleaved by the P450 side-chain cleavage enzyme (P450_{scc}) to generate pregnenolone, which serves as the template for all steroids (Goodman, 1994). The actual transfer of cholesterol from the outer to inner membrane of mitochondria has been determined to be the rate-limiting step in steroid synthesis. This step is mediated by the steroidogenic acute regulatory protein (StAR), which has been found in all steroidogenic tissues (reviewed in Stocco, 2001). In the adrenals ACTH induces StAR synthesis, which is initially expressed as a 37 kD protein followed by post-translational processing to 30 kD after activation by cAMP (Artemenko et al., 2001). The 30 kD protein is the active form of StAR and is responsible for shuttling cholesterol through the mitochondrial membranes so that cholesterol can be modified into pregnenolone by P450_{scc} (Stocco, 2001).

Hypothalamic or adrenal ablation has been shown to disrupt hibernation (Popovic et al., 1957; Ruby et al., 1996; Satinoff, 1967; Vidovic and Popovic, 1954), thus indicating the potential importance of the HPA axis to the initiation and maintenance of the hibernating state. Therefore, since StAR activity is

a rate-limiting component of the HPA axis, we have hypothesized that adrenal StAR expression would show seasonal variation in hibernating ground squirrels. The data presented here shows that StAR mRNA and protein expression in adrenals varies throughout the hibernation season in a manner that correlates with the hibernation phenotype.

Methods

Animals

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were purchased from TLS Research (Bartlett, Illinois). From April to August, animals were housed at 23° C with a 12:12-hour light/dark schedule and provided rodent chow and water *ad libitum*. In September, animals were moved to a temperature and light controlled chamber where the temperature was stepped down to 17° C in September and 11° C in October while maintaining the same conditions of food, water, and lighting. In November the temperature was fixed at 5° C with continual darkness and the absence of food. Animals hibernated from November until March with regular interbout arousals (IBAs; Pengelley and Fisher, 1961). At specific times, shown in Figures 1 and 2, animals were sacrificed by decapitation and organs and tissues were surgically removed, immediately frozen, and stored in liquid nitrogen.

RNA Isolation

RNA was isolated from tissues using the Qiagen RNeasy Mini kit (Valencia, CA) according to manufacturer's protocol. RNA was quantified by UV spectrophotometry and its quality was analyzed using an Agilent Bioanalyzer (Palo Alto, CA).

RT-PCR

One µg of total adrenal RNA from various animals was reverse transcribed using a Roche First Strand cDNA Synthesis kit (Indianapolis, IN). PCR was performed using LightCycler version 3.5 (Roche) with the Roche Faststart Master SYBR Green kit and StAR-specific primers. The internal standard for semiquantitative PCR was generated from a thirteen-lined ground squirrel testis StAR PCR product that was excised from a 1.5% agarose gel and purified using Qiaex II Gel Extraction kit (Qiagen). The sequence of the StAR PCR product was verified by automated sequencing using an ABI 377 DNA fragment analyzer at the University of Minnesota Advanced Genetic Analysis Center. Following PCR and

template quantification, samples were analyzed on a 1.5% agarose gel to verify correct sizing of the PCR product.

Western Blots

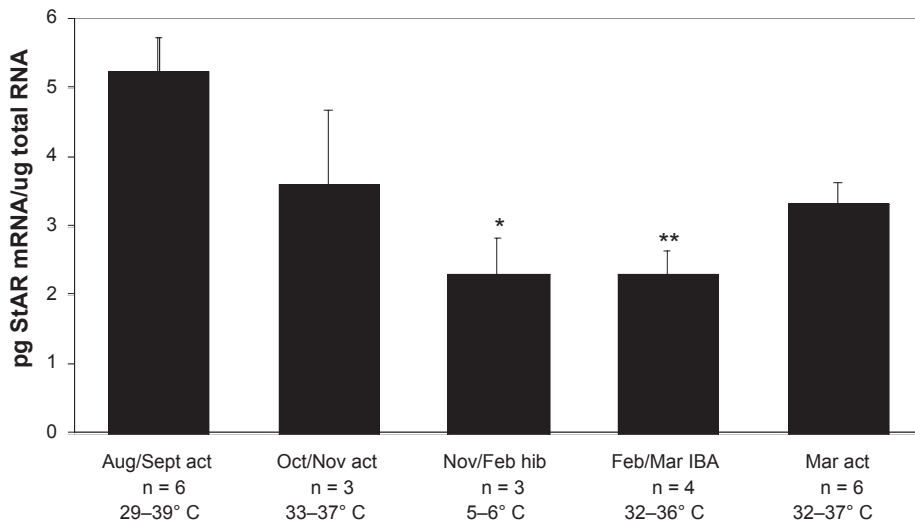
Anti-StAR antibody was raised against mouse StAR protein and was a kind gift from D. B. Hales (Artemenko et al., 2001). The β -actin antibody was obtained from Abcam and secondary antibodies were obtained from Promega. Protein was harvested by homogenizing adrenals in buffer containing 20 mM Tris pH 7.5, 40 mM NaCl, 1 mM DTT, and a protease inhibitor cocktail (Roche). The protein content of the homogenate was determined using the Pierce BCA kit. Total soluble protein was separated by SDS-PAGE on either 15% polyacrylamide gels or 12.5% Tris-HCl Criterion Precast gels (BioRad), and electrophoretically transferred to nitrocellulose (0.2 μ m). Blots were blocked, incubated in primary antibody (1:10,000 dilution), washed, and incubated in peroxidase-conjugated secondary antibody (1:10,000–1:20,000 dilution) as described previously (Buck et al., 2002). Protein bands were visualized by incubating with enhanced chemiluminescence peroxidase substrate (Pierce) followed by exposure to X-ray film. Densitometry was performed using an Epson Expression 800 flatbed scanner and NIH Image software.

Statistical Analysis

Significance at the $P < 0.05$ level was determined by one-way ANOVA using Microsoft Excel software.

Results and Discussion

The level of mRNA encoding the StAR protein was measured in adrenals to investigate whether a rate-limiting step of the HPA axis changes during hibernation. Primers specific for StAR mRNA were based on conserved StAR cDNA sequences from other mammals. These primers were used in reverse transcriptase/PCR reactions to amplify fragments of thirteen-lined ground squirrel StAR cDNA from adrenals and testis. These amplified cDNA fragments were then sequenced to confirm their identity. After confirming specificity, a StAR primer pair was used to determine relative levels of StAR mRNA in adrenals (Fig. 1). StAR mRNA concentration steadily declines from late summer through autumn and shows significantly lower levels during hibernation. Low levels are also seen during interbout arousals (IBAs), followed by an increase in StAR mRNA one week after spring arousal in March. The depression of adrenal StAR mRNA con-



*Fig. 1. RT-PCR analysis of adrenal StAR mRNA levels in thirteen-lined ground squirrels. Semiquantitative real-time RT-PCR analysis was performed using Roche LightCycler technology. Relative quantities of adrenal StAR mRNA, in relation to total adrenal RNA, were measured on different animals throughout the hibernation season. Month, state of activity, and body temperature at time of sacrifice are indicated below each bar. Number of animals analyzed (n) is shown for each point. Statistically significant comparisons to active August/September StAR mRNA levels were determined by one-way ANOVA; * $P = 0.01$, ** $P = 0.008$. Abbreviations: act = active, hib = hibernating, IBA = interbout arousal.*

centration appears to mirror the profile of serum cortisol levels in mammalian hibernators in late summer/early autumn (Kenagy and Place, 2000) and during hibernation (Shivatcheva et al., 1988).

Western blot analysis using antibody raised against mouse StAR protein was used to determine the size and relative amount of StAR throughout the hibernation season (Fig. 2). The mass of thirteen-lined ground squirrel StAR protein in adrenals was measured at 29 kD (Fig. 2A). This measurement is very close to the biologically active 30 kD form identified in other mammals (Artemenko et al., 2001; Stocco, 2001). We did not detect the unprocessed 37 kD form in ground squirrel adrenals.

Beginning with September active ground squirrels, high concentrations of StAR show a gradual decline in October active and October/November hibernating animals in relation to total adrenal protein (Fig. 2A). StAR protein levels in November/December hibernating and November/December IBA animals are significantly lower than those in September active ground squirrels in relation to adrenal β -actin (Fig. 2B). Significantly lower StAR levels are also seen one week after spring arousal in mid-March. This depression of StAR protein

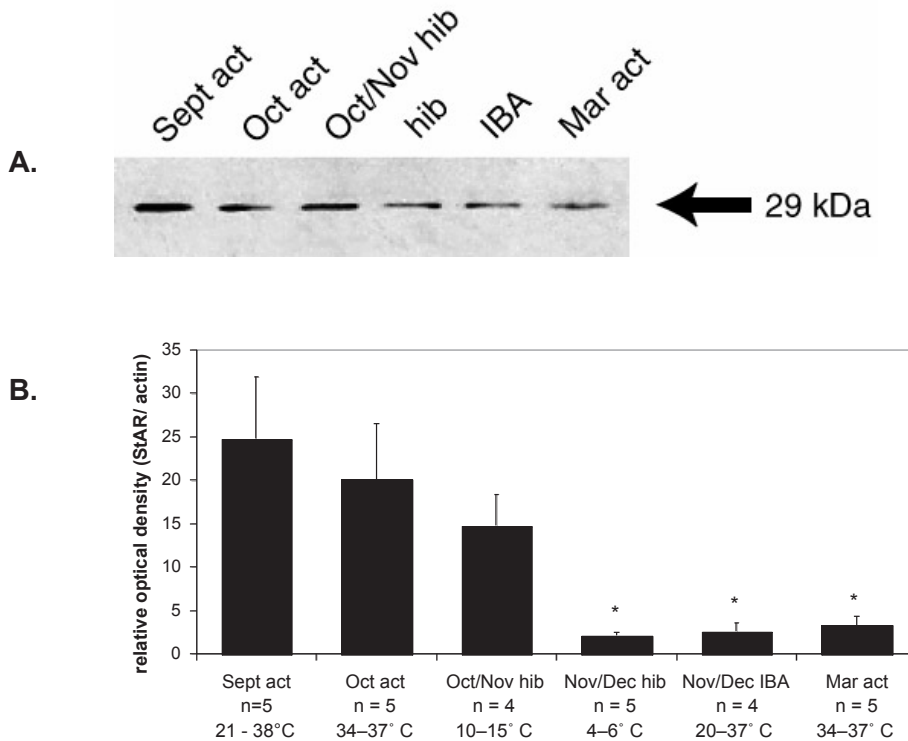


Fig. 2. Western blot analysis of StAR protein levels in thirteen-lined ground squirrel adrenals. (A) A representative blot showing the size of StAR protein based on molecular weight markers. (B) Densitometry of Western blots using anti-StAR antibodies reveals trends in StAR expression versus β -actin expression throughout the hibernation season. Month, state of activity, and body temperature at time of sacrifice are indicated below each bar. Number of animals analyzed (*n*) is shown for each point. Statistically significant comparisons to September StAR protein levels were determined by one-way ANOVA; **P* < 0.05. Abbreviations: act = active, hib = hibernating, IBA = interbout arousal.

during hibernation and IBAs resembles changes in transcript levels and suggests down-regulation of StAR activity during hibernation. Correlation of changes in levels of mRNA and protein at low body temperatures during hibernation have also been seen in multiple tissues for pyruvate dehydrogenase kinase isoenzyme 4 (PDK4; Buck et al., 2002) and pancreatic triacylglycerol lipase (PTL; Squire et al., 2003).

Based on our analysis of StAR expression, Fig. 3 is a model showing the proposed role of StAR protein in controlling hibernation via the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus region of the brain integrates external stimuli and internal signals to monitor satiety, circadian rhythms, ambient temperature, and light-dark cycles. Corticotropin-releasing hormone (CRH) produced by the hypothalamus acts on the pituitary gland to elicit secretion of adrenocorticotrophic hormone (ACTH). ACTH binds receptors in adrenocortical cells and induces synthesis of steroids, including glucocorticoids, via the

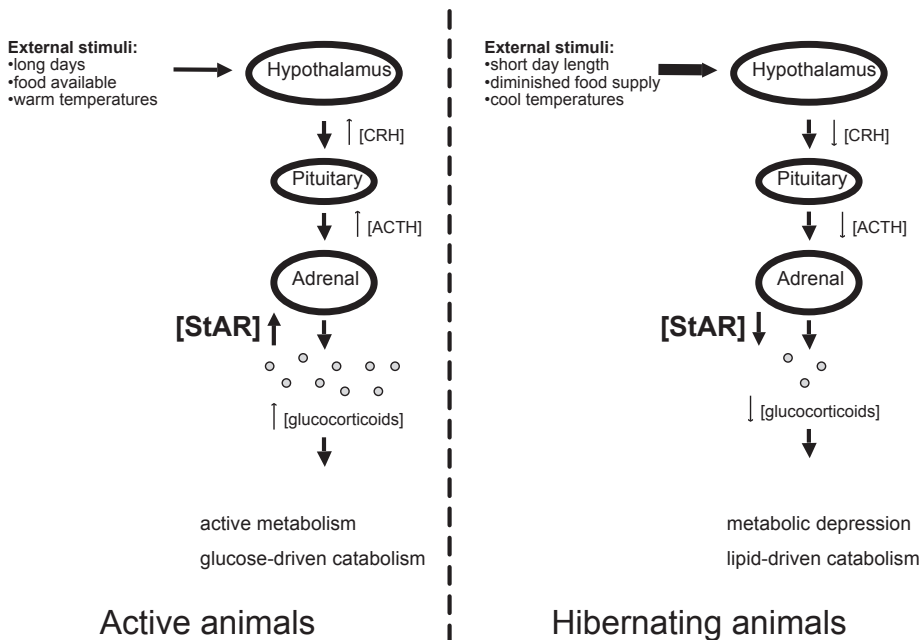


Fig. 3. Model showing proposed role of HPA axis and StAR activity before and during hibernation. Vertical arrows adjacent to CRH, ACTH, StAR, and glucocorticoids indicate changes in their respective activity or concentration.

adrenal StAR protein. Based on our results, summer animals have an elevated StAR concentration, resulting in higher glucocorticoid levels that give rise to an active, glucose driven metabolism. During hibernation, adrenal StAR levels are depressed, resulting in metabolic rate reduction, lipid driven metabolism, and torpor. Further studies on the role of the StAR protein during hibernation will require a focus on the mechanism controlling hypothalamic hormone secretion, the production of adrenal steroids by StAR, and the effects of these hormones on the hibernation phenotype.

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A Quest for the Origin of Mammalian Uncoupling Proteins

MARTIN JASTROCH,¹ SIGRID STÖHR,¹ KERRY WITHERS,² AND MARTIN KLINGENSPOR¹

¹ Philipps University of Marburg, Department of Biology, Animal Physiology, Germany

² Department of Biological and Physical Sciences, University of Southern Queensland, Toowoomba, Queensland, Australia

Abstract. Nonshivering thermogenesis is dependent on the presence of UCP1 in brown adipose tissue, whereas the function of UCP2 and UCP3 is most likely related to mitochondrial superoxide metabolism and/or fatty acid oxidation. These three members of the core UCP family are known in eutherians but had not been found in marsupials and monotremes so far. The objective of our search is to determine the origin of UCP1 and “classical” nonshivering thermogenesis. Furthermore, our approach to characterize UCP2/UCP3 in distantly related animal species will assist in the functional annotation of these proteins.

We recently reported on the molecular identification, tissue-distribution, and physiological regulation of UCP2 and UCP3 mRNA in the marsupial *Antechinus flavipes* (yellow-footed Antechinus). Despite separate evolution of the marsupial lineage since 130 million years, our data suggest a conserved physiological role of these UCPs. Here, we present the immunological detection of marsupial UCP3 in skeletal muscle using antibodies raised against mouse/rat UCP3. A comprehensive phylogenetic analysis led us to hypothesize that all uncoupling proteins were already present at the evolutionary stage of modern teleost fishes and questions the unique presence of UCP1 in placental mammals. However, the search for UCP1 in nonplacental mammals has been unsuccessful so far and is most likely hampered by the more rapid evolution of UCP1 as compared to other UCPs.

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The Quest for UCP1 in Nonplacental Mammals

In placental mammals, uncoupling protein 1 (UCP1) is responsible for non-shivering thermogenesis in brown adipose tissue mitochondria. The protein is inserted with six α -helical transmembrane domains into the inner mitochondrial membrane and catalyzes proton flux into the matrix. Protonmotive force normally driving ATP synthesis is thereby dissipated as heat. In mice acutely exposed to the cold this unique mechanism of heat production is essential for survival (Enerbäck et al., 1997), and in small placental hibernators, brown adipose tissue mass is increased in relation to body mass as compared to nonhibernating mammals (Heldmaier, 1971). In the past, physiologists, morphologists, and biochemists have repeatedly questioned the common view that brown adipose tissue and UCP1 are monophyletic traits of placental mammals. In some marsupial species, treatment with sympathomimetics increased resting metabolic rate, resembling the adrenergic stimulation of nonshivering thermogenesis in brown adipose tissue of placental mammals (Loudon et al., 1985), but this finding was not confirmed in other studies. The interscapular fat deposit of Bennett's wallaby consists of multilocular adipocytes (Loudon et al., 1985), but so do white adipocytes in cold-stressed placental mammals (Loncar et al., 1988). The brownish appearance of the interscapular fat depot in the small marsupial carnivores *Sminthopsis macroura* and *Antechinus flavipes* (unpublished observation) indicates a high tissue content of mitochondria, but only the identification of UCP1 in marsupial adipose tissue and the demonstration of uncoupled mitochondrial respiration would provide hard evidence for the existence of brown adipose tissue in marsupials.

It is well established that purine nucleotides bind to UCP1 and inhibit proton transport activity in the absence of free fatty acids (Klingenspor, 2003). Indeed, increased GDP-binding to interscapular brown adipose tissue mitochondria of the Bennett's wallaby suggested the presence of UCP1 (Loudon et al., 1985). Furthermore, UCP1-like immunoreactivity was reported in the interscapular fat deposit of *Sminthopsis crassicaudata* using an antibody raised against squirrel UCP1 (Hope et al., 1997). However, by using rodent probes or primers deduced from rodent UCP1 sequences, UCP1 mRNA could not be detected in the Tasmanian bettong (Rose et al., 1999) and the Tasmanian devil (Kabat et al., 2003). In our laboratory the attempts to identify UCP1 mRNA in marsupial adipose tissue with heterologous probes were futile as well. Previously, the use of a labelled oligomer from a conserved region of UCP1 had been successful to detect the mRNA in a variety of placentals (Brander et al., 1993). However, in our

hands several attempts to amplify marsupial UCP1 by RT-PCR using different primers deduced from conserved regions of the UCP1 coding sequence failed. Taken together, the monophyletic nature of the UCP1 gene in placental mammals has not been seriously challenged, as sequence data providing unambiguous proof for the presence of UCP1 in nonplacental mammals have not been reported.

Identification of Uncoupling Proteins in Marsupials

Even in tissues other than brown adipose tissue, a significant portion of mitochondrial respiration is due to proton leakage across the inner mitochondrial membrane but the biochemical cause for this leak has not been resolved (Rolfe and Brown, 1997). In 1997, paralogous proteins similar to UCP1 were found (Boss et al., 1997; Fleury et al., 1997). Because of their sequence and structural similarity, and their uncoupling activity in certain experimental conditions, they were named UCP2 and UCP3. In placental mammals UCP2 is ubiquitously expressed in multiple tissues, whereas UCP3 is restricted to skeletal and heart muscle. Pertaining to their phylogenetic distribution, UCP2 and UCP3 are described in placental mammals. Moreover, UCP2 is also found in fish (Stuart et al., 1999) and a UCP-like protein is present in birds (Raimbault et al., 2001). None of the core UCP family members have been identified so far in invertebrates.

A thermogenic function of UCP2 and UCP3 in brown adipose tissue is unlikely since they do not compensate for defective thermogenesis in UCP1 ablated mice, and ablation of the UCP2 or UCP3 gene does not impair energy balance, cold resistance, or nonshivering thermogenesis in rodents (Cannon and Nedergaard, 2004). However, a recent study suggests a possible thermogenic activity of UCP3 in skeletal muscle of mice in response to extasy treatment (Mills et al., 2003). Accumulating evidence suggests that UCP3 may play a role in mitochondrial lipid metabolism operating as a fatty acid anion exporter (Himms-Hagen and Harper, 2001; Schrauwen et al., 2003). Other nonthermogenic-uncoupling functions have been assigned to UCP2, including the reduction of superoxide generation in mitochondria by mild uncoupling, diminished pancreatic insulin secretion by lowering the cytosolic ATP/ADP ratio, neuroprotection and modulation of the immune system (Nedergaard and Cannon, 2003).

We cloned full-length marsupial UCP2 and UCP3 cDNAs in *Antechinus flavipes* and a UCP2 cDNA in *Sminthopsis macroura* (stripe-faced dunnart) (Jastroch et al., 2004). Initial hybridization analyses using heterologous probes from mouse and hamster revealed the presence of the UCP2 gene and transcript

but not UCP1 and UCP3. Since the marsupial UCP3 sequence was not detectable using heterologous rodent cDNA probes, we applied an alternative cloning strategy based on conserved blocks within the UCP3 coding sequence. We deduced consensus oligomers from placental UCP3, also including bird UCP. We suggest that the latter is most likely the avian orthologue of UCP3, which is supported by the physiological regulation of gene expression (Evock-Clover CM et al., 2002) and phylogenetic relation to mammalian UCP3 (Fig. 3). We amplified a 480 bp cDNA fragment from skeletal muscle cDNA of *A. flavipes*, which we used to isolate a full-length UCP3 clone (Genbank Acc. #AY519198) from a cDNA library.

In *A. flavipes* exposed to 5° C for two days, the mRNA levels of UCP2 and UCP3 in interscapular adipose tissue and in skeletal muscle were not increased (Jastroch et al., 2004), which appears to exclude a potential role in adaptive thermogenesis in marsupials. However, we recently achieved the immunological detection of UCP3 in protein extracts from skeletal muscle (Fig. 1). UCP3 protein levels in cold-exposed *A. flavipes* were elevated nearly 2-fold. In contrast to the mRNA data, this finding suggests a role of UCP3 for muscle thermogenesis.

We also studied the regulation of UCP2 and UCP3 mRNA in response to two days of food deprivation. Whereas UCP2 mRNA remained unchanged in most tissues investigated, UCP3 mRNA was upregulated 6-fold in heart and 2.5-fold in skeletal muscle. This mode of physiological regulation is consistent with the upregulation of UCP3 expression in skeletal muscle of rodents induced by fasting but not compatible with a thermogenic function of UCP3. Since muscle energy expenditure in fasted animals is lowered, the efficiency of mitochondrial ATP synthesis is unlikely to be reduced in this negative state of energy balance.

When Did the UCP1 Gene Emerge in Vertebrate Evolution?

One hundred thirty million years of evolution separated marsupials and eutherian mammals, leading to sequence variations in the UCP genes that may impose some difficulties in their identification in marsupials. In this respect, the search for UCP2 orthologues is less difficult due to the high sequence conservation in distantly related taxa, such as amphibians, fish, and rodents. In retrospect, the high identity of rodent and marsupial cDNA sequences (90%) facilitated detection of the marsupial gene and the corresponding mRNA by heterologous hybridization assays. However, for UCP3 this approach completely failed due to only 80% global identity between marsupial and rodent UCP3. The search for regions in the coding sequence conserved across phylogenetically distinct species

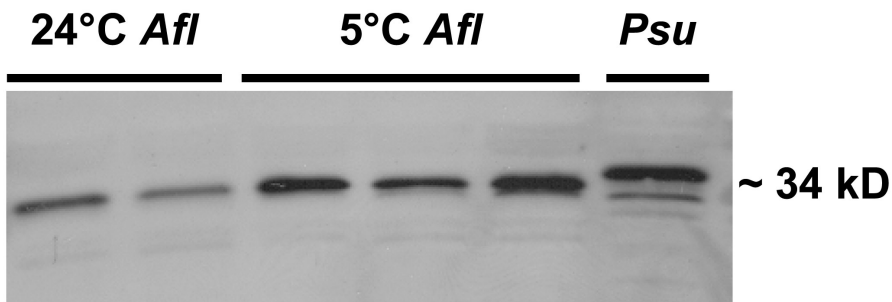


Fig. 1: Immunological detection of UCP3 in cold and warm acclimated yellow-footed antechinus (*Antechinus flavipes*, Afl). Forty micrograms of protein from skeletal muscle per lane were subjected to Western blot analysis using an antibody against mouse/rat UCP3. An equal amount of skeletal muscle protein from the Djungarian hamster (*Phodopus sungorus*, Psu) served as a control.

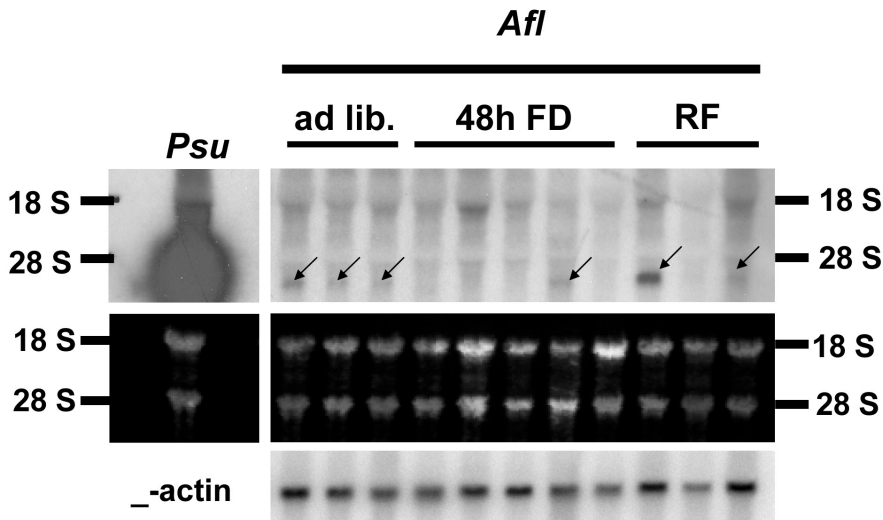


Fig. 2: Northern blot of skeletal muscle from fed, fasted, and refed *A. flavipes*. Twenty micrograms of total RNA were analysed by Northern blotting with a rat UCP1 cDNA probe and β -actin probe. Arrows indicate the apparent detection of marsupial UCP1. Afl: *Antechinus flavipes*, Psu: *Phodopus sungorus*, ad lib.: ad libitum, FD: food-deprived for 48 hours, RF: refed for 24 hours.

led to successful PCR amplification of a marsupial UCP3 cDNA fragment. Notably, the full-length marsupial UCP3 cDNA probe did not hybridize with hamster UCP3 mRNA on Northern blots of total skeletal muscle RNA, whereas a clear albeit weaker hybridization signal was observed probing hamster spleen RNA with the marsupial UCP2 cDNA (Jastroch et al., 2004). This illustrates the limitations of heterologous hybridization assays in the search for orthologues in distantly related species.

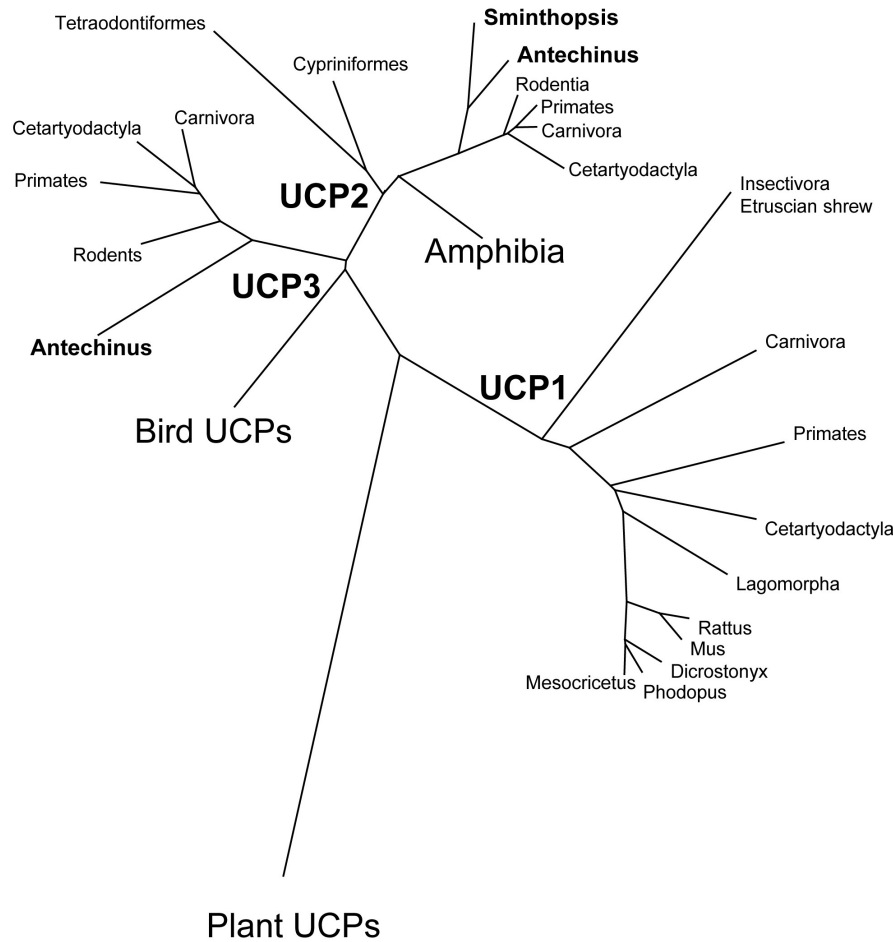


Fig. 3: Unrooted phylogenetic tree of the core UCP family using the neighbor-joining method.

Knowing this, we definitely cannot conclude that nonplacental mammals lack the UCP1 gene. We speculate that the search for marsupial UCP1 was not successful yet, since the sequence similarity to eutherian UCP1 can be expected to be rather low. Even within eutherian mammals, the comparison of UCP1 orthologues from phylogenetic distant taxa, as exemplified by comparison of Etruscan shrew UCP1 and mouse UCP1, reveals only 75% global identity (based on 245 amino acids known from shrew). Klaus and colleagues reported that the signal intensity for Etruscan shrew UCP1 mRNA is strongly diminished when using a heterologous rodent probe for hybridization analysis (Klaus et al., 1996). This indicates a rapid evolution of this protein within the eutherian infraclass and certainly complicates straightforward attempts to identify the eutherian orthologue of UCP1 in nonplacental mammals.

Despite these theoretical considerations, we screened multiple tissues sampled from fed, fasted, and refed *A. flavipes* by Northern blot analysis for the presence of UCP1 mRNA with a full-length radio-labelled rat UCP1 cDNA probe. Surprisingly, in skeletal muscle of several individuals we observed a barely detectable hybridization signal when Northern blots were washed at low stringency (indicated by arrows in Fig. 2). This apparent detection of marsupial UCP1 mRNA in skeletal muscle did not depend on feeding state and may rather be related to life history of the animals or variation in tissue sampling. Rehybridization of the same blot with UCP2 and UCP3 definitely excluded possible cross-hybridization, as judged from transcript size. Screening of skeletal muscle cDNA libraries will reveal whether the detected transcript indeed represents marsupial UCP1.

Regarding the phylogenetic tree of all known UCPs, it is well possible that UCP1 not only occurs in placental mammals. Using different outgroups, e.g., plant UCPs (Fig. 3), brain mitochondrial carrier protein (BMCP1), or the oxalacetate-malate carrier, the branching of UCP1 occurs at least at the evolutionary stage of modern teleost fish. Thus, UCP1 and UCP3 may also be found in ectothermic vertebrates unless these genes went extinct in the living species.

Orthologues are defined as proteins sharing common ancestry and function. However, if an uncoupling protein gene sharing a common ancestor with mammalian UCP1 does exist in ectothermic vertebrates, a global thermogenic function appears unlikely. The thermogenic function may rather represent a new property of UCP1 that evolved in the mammalian lineage. Despite the fact that UCP1 is regarded as a marker protein for brown adipose tissue in eutherian mammals, the expression of this protein if present might not be restricted to

adipose tissue in nonplacental mammals and ectothermic vertebrates. Notably, an involvement of UCP1 in intestinal relaxation was suggested by a study in UCP1-ablated mice (Shabalina et al., 2002), adding to the ongoing discussion as to whether the UCP1 gene is also expressed in smooth longitudinal muscle of rodents (Nibbelink et al., 2001; Rousset et al., 2003).

We therefore conclude that the search for UCP1 in nonplacental mammals has to be intensified on the molecular level.

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Brown-Fat-Derived and Thyroid-Hormone Thermogenesis: Mechanisms and Interactions

JAN NEDERGAARD, VALERIA GOLOZOUBOVA, AND BARBARA CANNON
Wenner-Gren Institute, the Arrhenius Laboratories F3, Stockholm University, SE-106 91
Stockholm, Sweden

Abstract. Although many metabolic processes are thermogenic, only shivering thermogenesis and classical nonshivering thermogenesis are mechanisms of purposeful heat production. The thermogenic mechanism in classical nonshivering thermogenesis is fully dependent on the presence of the uncoupling protein UCP1 in brown adipose tissue. In all other “thermogeneses,” such as what is referred to as thyroid thermogenesis, the heat must be considered waste heat from other processes. A “thermogenic mechanism” may therefore not exist in these cases. However, both through the thermoregulatory effects of thyroid-hormone-induced heat production, and through direct thyroid-hormone effects within brown adipose tissue, thyroid hormones influence brown adipose tissue-derived thermogenesis. Studies on animals without thyroid-hormone receptors have been helpful in clarifying these interactions.

Thermogenesis

All ongoing metabolism is thermogenic, but we tend in interpretation to discriminate between thermogenesis as an unintentional but obligatory side effect of otherwise ongoing processes in the body (i.e., waste heat), and thermogenesis that has heat production as its purpose, i.e., thermoregulatory thermogenesis. Since we cannot directly read off the purpose of biological events, this distinction is in reality not easily made.

True (i.e., thermoregulatory) thermogenesis is thus only present when it is needed, i.e., under circumstances where extra heat is needed, in addition to

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waste heat, to defend body temperature. It is therefore necessarily facultative, i.e., it is turned on and off in response to alterations in environmental temperature. Only two types of thermogenesis fulfil this demand: shivering thermogenesis, and nonshivering thermogenesis in its classical, restricted sense of a thermogenesis that replaces shivering in the cold. All other types of metabolism that have heat production as one outcome can hardly be considered examples of true thermogenesis; nevertheless, they are often referred to as such, in formulations such as diet-induced thermogenesis, thyroid (hormone) thermogenesis, and even basal thermogenesis. Concerning all these other types of waste-heat thermogenesis (i.e., all others than shivering and classical nonshivering thermogenesis), we have not primarily to seek for their “thermogenic mechanism” but to understand how the thermogenesis observed may be an obligatory but unintentional outcome of the processes.

We shall here illustrate this tenet by comparing the brown-fat-derived thermogenesis with thyroid (hormone) thermogenesis and examining their interaction.

Brown-fat Derived Thermogenesis

Brown-fat-derived Thermogenesis is UCP1-dependent Thermogenesis

Muscle is evidently the tissue that is fully responsible for the heat production in *shivering* thermogenesis (for the principal relation between shivering thermogenesis, nonshivering thermogenesis and UCP1, see Fig. 1). That brown adipose tissue is the tissue that is fully responsible for *classical nonshivering thermogenesis* is an insight that has only successively matured, and opinions are still found that a nonshivering thermogenesis located elsewhere than brown adipose tissue may exist.

Heat production of brown adipose tissue is fully due to the activity of the archetypal uncoupling protein, UCP1 (thermogenin) (reviewed in Nedergaard et al., 2001). Indeed, without UCP1, brown-fat cells are unable to demonstrate norepinephrine-induced thermogenesis (Matthias et al., 2000). Thus, in brown-fat cells there is no other mechanism than UCP1 that is responsible for heat production, nor can any other mechanism be compensatorily activated in this tissue in order to produce heat.

Although there is ample evidence that the heat production in brown adipose tissue is due to UCP1 activity, a full agreement on how UCP1 functions to actually allow for heat production has still not been attained. Suggested explanations for the heat-dissipating process of UCP1 include futile fatty acid cycling, but an uncoupling through such a process can be demonstrated for several types of mitochondrial carrier proteins, and the evolutionary advantage gained by the

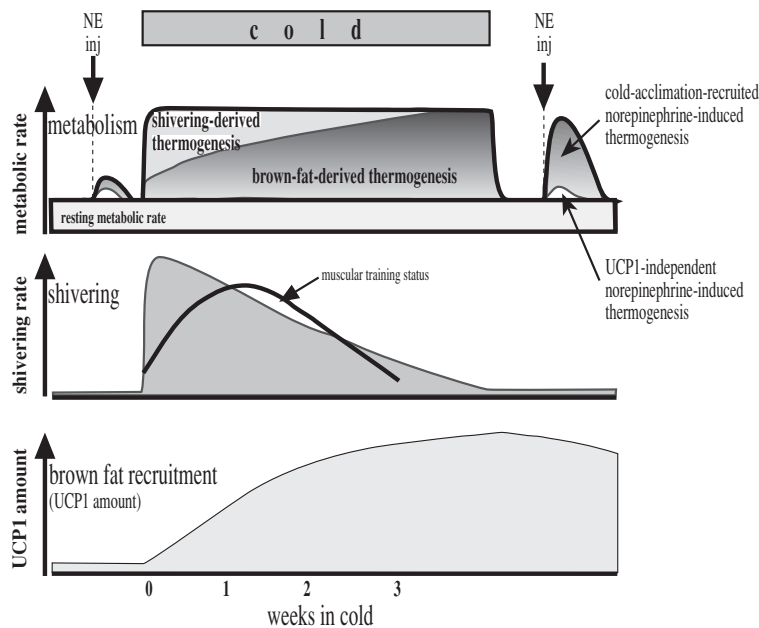


Fig. 1. The relationship between total metabolic rate, shivering thermogenesis, non-shivering thermogenesis, norepinephrine-induced thermogenesis, and UCP1 amount during cold acclimation. This is a principal summary of established patterns. The animal is thought to first be living at its thermoneutral temperature where its total metabolic rate is equal to its resting metabolic rate (top panel). When here injected with norepinephrine (NE), a small metabolic response is seen that upon analysis can be divided into a UCP1-independent and a UCP1-dependent part. When the animal is transferred to the cold, it will immediately increase its metabolism, initially mainly by shivering. However, as a consequence of successive recruitment of brown adipose tissue thermogenic capacity (displayed in lower panel), shivering intensity will successively decrease (middle panel). Constant shivering may be considered a muscle training condition, and effects of muscle training (e.g., increased muscular oxidative capacity) are transiently observed (displayed in middle panel). When the cold-acclimated animal is retransferred to the warmth, its metabolism immediately decreases (top panel), but since its brown adipose tissue still is much recruited (lower panel), the response to a norepinephrine injection is now much higher. Analysis of this response indicates that the UCP1-independent response is unaffected by acclimation to cold; it is thus the UCP1-dependent part that is responsible for the increased response to norepinephrine. (The figure is modified from Cannon and Nedergaard, 2004, where a more complete analysis is given.)

development of UCP1 is thus not evident. Some experiments would instead indicate that UCP1 works directly as a regulated H^+ (or OH^-) pore/channel (reviewed in Nedergaard, et al., 2001). Investigations from different laboratories have recently indicated substances such as ubiquinone, superoxide, and superoxide products such as nonenal, as activators of all uncoupling proteins, i.e. not only UCP1 but also UCP2/UCP3. However, since UCP1 has sequence characteristics not found in UCP2/UCP3, it is surprising if regulation and activity of all these proteins are identical.

Nonshivering Thermogenesis is Fully Due to Brown Adipose Tissue Activity

UCP1 is fully responsible for classical nonshivering thermogenesis. Animals without UCP1 continue to shiver in the cold and never develop nonshivering thermogenesis (Golozoubova et al., 2001). This also means that no other proteins, not even the other UCPs, can substitute for UCP1 in this function. UCP1 is thus the only protein with classical nonshivering thermogenesis as its function; it is probably also the only protein with mitochondrial uncoupling as its function. The “new” uncoupling proteins, UCP2 and UCP3, were so named due to their sequence similarity to UCP1 (the so-called UCP4 and UCP5 are not even more similar to UCP1 than they are to other mitochondrial carrier proteins). There is presently no indication that UCP2/UCP3 are involved in processes related to purposeful thermogenesis, and ideas that they may be involved in lowering of metabolic efficiency (which would have thermogenesis as a byproduct) have not been substantiated. The tenet that they physiologically function as uncoupling proteins carries little if any experimental evidence (see review Nedergaard and Cannon, 2003).

The lack of development of any nonshivering thermogenesis in UCP1-ablated mice also means that *muscle nonshivering thermogenesis does not exist*, i.e., no facultative and adaptive mechanism located to muscle can take over if UCP1 is not present. This does not, of course, exclude that muscles participate significantly in “basal metabolism.” Mitochondrial proton leak, corresponding to “state 4,” is believed to contribute significantly to the “basal” metabolic rate of the muscle. A transition from fast-twitch to slow-twitch muscle (white to red) is accompanied by an increased amount of mitochondria. An increase in total amount of slow-twitch muscle would therefore be predicted to result in an increased “basal” metabolic rate. Thus, if prolonged shivering leads to recruitment of slow-twitch muscle, an adaptive increase in basal metabolism may be expected to occur; this increase would, however, not be facultative, and it would only cor-

respond to a minor component in total heat needed in the cold. However, experimentally, we have seen no evidence that the basal metabolism is increased in the constantly shivering UCP1-ablated mice (Golozoubova et al., 2001). Thus, if such a mechanism exists, its magnitude would seem to be such that it is not measurable as a component of total metabolism.

Norepinephrine-induced Thermogenesis

In parallel with the adaptive development of nonshivering thermogenesis in cold-acclimating animals (Fig. 1), these animals exhibit an increased metabolic response to injections or infusions of norepinephrine (Fig. 1, top panel). Earlier investigations have indicated that brown adipose tissue is responsible for 60–80% of the total oxygen consumption during norepinephrine-induced thermogenesis in cold-acclimated animals (Foster and Frydman, 1978). These statements have left open the possibility that other processes than the heat production of brown adipose tissue could be involved in norepinephrine-induced thermogenesis. However, in UCP1-ablated mice, there is no increase at all in the response to norepinephrine as a consequence of cold acclimation (summarized in Nedergaard, et al., 2001). Thus, the heat production that cannot be ascribed directly to brown adipose tissue in wild-type animals is clearly waste heat from the action of accessory organs, i.e., the lung and the heart, as originally suggested (Foster and Frydman, 1978). Although this heat in its origin is waste heat, it is, of course, functionally equivalent to purposeful heat and helps the animal to combat the cold to the same extent as heat derived from brown adipose tissue.

UCPI and Diet-induced Thermogenesis

When animals overeat (high-fat diets or “cafeteria diet”), they display an increased metabolic rate. This is commonly referred to as diet-induced thermogenesis. Here, the thermogenesis observed is not purposeful heat but is the waste heat necessarily released when excess food is being combusted in order to reduce metabolic efficiency. It is generally assumed that brown adipose tissue is also involved in the mechanism leading to diet-induced thermogenesis. However, the picture here is not so simple as it is concerning classical nonshivering thermogenesis. It is clear that the increase in norepinephrine-induced thermogenesis observed in wild-type animals exposed to a recruiting diet is not seen in animals without UCP1 (summarized in Cannon and Nedergaard, 2004). However, exposure to recruiting diets is also associated with an increased body temperature, in the order of 1° C, in animals both with and without UCP1. The mechanism

behind this body temperature increase is not known; it is often considered a hyperthermia. However, this cannot be the case since the increased body temperature is readily observed in animals living below the so-called lower critical temperature where the animals have ample possibility to dissipate the extra heat and defend their normal body temperature. It is therefore a fever (or a hyperpyrexia if the word "fever" is restricted to infection-related hyperpyrexias). With a Q_{10} of about 3, a 1° C change in body temperature would, providing that the animals behave as chemical machines, result in a 10% increase in metabolic rate. This is thus a type of "delocalized" thermogenesis.

Thyroid Thermogenesis

Thyroid (hormone-induced) thermogenesis is not facultative, i.e., it is not switched on and off as a direct response to cold or warm. Thyroid thermogenesis is therefore not relevant as a mechanism for nonshivering thermogenesis *sensu strictu* ("cold-acclimation-recruited, cold-induced nonshivering thermogenesis"). It is thus probably inherently unfruitful to search for "the mechanism of the thermogenesis" underlying thyroid thermogenesis; the thermogenesis is probably waste heat from processes initiated for other purposes. What these are is still not understood. In a broad sense, thyroid hormone(s) may be said to regulate the metabolic activity of the body, and the heat produced will thus be waste heat from increased metabolism. A stimulatory effect of thyroid hormone on oxygen consumption is observed also in poikilotherms such as reptiles (John-Adler, 1983), and since reptiles do not defend their body temperature metabolically, the increased metabolism cannot have thermogenesis as its purpose. Rather, one can perhaps think of increased thyroid hormone levels leading to an increased metabolic capacity, broadly regulating metabolic capacity in relation to energy availability. This would at least seem to have been the original function of thyroid hormone. However, since thyroid hormone does increase oxidative rate, i.e., it is functionally thermogenic (even though it probably does not have thermogenesis as its purpose), and since any such thermogenesis must necessarily diminish the need for facultative thermogenesis, thyroid thermogenesis must influence thermogenesis originating in brown adipose tissue.

A Possible Central Mechanism for Thyroid Thermogenesis

Hyperthyroid animals display an increased body temperature. This is often referred to as thyroid hormone-induced hyperthermia, but because the increased body temperature is observed even below the thermoneutral zone, it must be

a hyperpyrexia (fever) (Székely, 1970), similar to what is seen in diet-induced thermogenesis. Correspondingly, the absence of all thyroid hormone receptors leads to a decreased body temperature set-point of about 1° C (Golozoubova et al., 2004). If again a chemical machine-concept of body metabolism is presumed, the several °C difference in body temperature between the hypothyroid and the euthyroid state would in themselves correspond to an increased metabolism, amounting to about 10% per degree. This “thermogenesis” would thus be a delocalized thermogenesis where no specific tissue is involved. Because an increased body temperature is normally associated with an increased general activity, determination of general activity level may be (one of) the real functions of thyroid hormones, and the increased thermogenesis may then simply be understood as an increased waste heat production due to the increased metabolism. It then has no purpose as such, and has no specific mechanism.

Suggested Peripheral Thermogenic Mechanisms

The centrally regulated thermogenic effect of thyroid hormone discussed above does not require any increased amount of enzymes; the enzymes present simply work at a higher speed due to the increased temperature. However, treatment with thyroid hormone increases the gene expression or the amount of several enzymes. Although these increases are often considered to be part of the “thermogenic mechanism,” they may instead be considered reflections of the original function of thyroid hormone, i.e., as a regulator of development and tissue differentiation. Further, in itself, an increased expression of an enzyme can hardly be conceived to be an explanation for thermogenesis. The enzymes discussed are often oxidative but an increased oxidative capacity, leading to increased ATP production, needs an increased ATP-utilizing mechanism to manifest itself as a thermogenesis. Even an increase in ATP-utilizing enzymes, such as the Na⁺/K⁺-ATPase, does not in itself lead to increased thermogenesis; in this case also an increased membrane permeability for ions is needed, and this has not been reported.

However, concerning two suggestions, true energy-dissipating mechanisms have been proposed. One is Ca²⁺ cycling in muscle. Thyroid hormone leads to an increase in the Ca²⁺ pumping SERCA enzyme, and it has been suggested, with experimental evidence, that when the Ca²⁺ gradient develops, Ca²⁺ handling due to this becomes inefficient, leading to heat production (Reis et al., 2002). It has also been observed that an increased proton leak occurs in mitochondria of thyroid hormone-treated animals, e.g., in liver mitochondria (Brand et al., 1992). In one of the original articles on UCP3, some evidence

was presented that this protein could be the mediator of thyroid thermogenesis (Gong et al., 1997). This suggestion can really be seen as a molecular formulation of the proton leak hypothesis. However, UCP3 is not expressed in liver where increased proton permeability has been observed in hyperthyroid animals, and the thermogenic effect of thyroid hormone is not abolished or diminished in UCP3-ablated animals (Gong et al., 2000). There is therefore no reason to think that UCP3 mediates thyroid thermogenesis.

Are There Nongenomic Metabolic Effects of Thyroid Hormones?

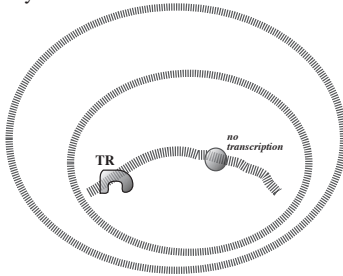
Although thyroid hormones are presently normally conceived as functioning through binding to the identified nuclear thyroid hormone receptors, there are experiments that have been interpreted to indicate that thyroid hormones (T_3 or T_4) or products of their deiodination (T_2) may induce metabolic effects not mediated via the genomic effects and thus probably not through the thyroid hormone receptors. Mice lacking all thyroid hormone receptors have a high circulating level of T_3 and T_4 (and possibly T_2) (this is due to the central feedback processes controlling thyroid hormone levels being dependent upon the presence of these thyroid hormone receptors). Thus, although these animals are highly hyperthyroidemic, there is no evidence for an increased metabolism in these mice (Golozoubova et al., 2004). Thus, the *in-vitro* observations on a nongenomic effect of thyroid hormones on thermogenesis can apparently not be replicated within an animal, and there is presently no reason to think that thyroid hormone works in any other way than through activating the established thyroid hormone receptors and then activating the corresponding gene expression.

Thyroid Hormones as Gene Activators

In contrast, it is likely that thyroid hormones perform all their functions through nuclear thyroid hormone receptors. The effects of thyroid hormone mediated via these receptors may be positive for gene expression (Fig. 2); alternatively, the thyroid hormone receptor as such (i.e., in the absence of thyroid hormone) can be a repressor of gene expression (discussed below and in Fig. 3). In the case of positively acting thyroid hormone receptors, the lack of thyroid hormone receptors should lead to phenotypes similar to hypothyroidism. Concerning thermoregulation, this is clearly the case for body temperature and basal metabolism (Golozoubova et al., 2004).

However, much more dramatic effects of thyroid hormones are seen when unliganded thyroid hormone receptors function as gene repressors. An example of this is described below with respect to brown adipose tissue.

Cell
in hypothyroid mouse



Cell
in euthyroid mouse

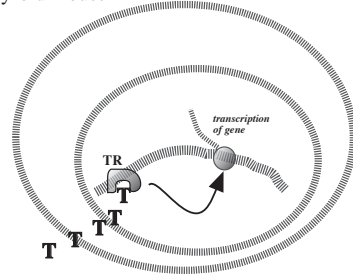
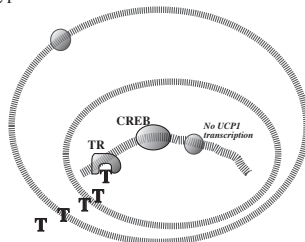
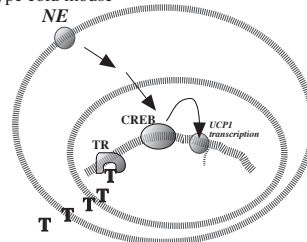


Fig. 2. Thyroid hormone (T) when it functions as a positive gene expression inducer by binding to a thyroid hormone receptor (TR). Genes induced may be peripheral and members of mitochondrial catabolic oxidative processes or ATP-utilizing processes such as the Na^+/K^+ -ATPase or the Ca^{2+} pumping SERCA enzyme. Genes induced may also be central; it would seem to be the case for genes involved in control of body temperature. Contrast the function of thyroid hormone as a gene expression repressor (Fig. 3).

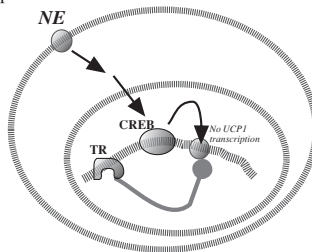
A. Brown fat cell in euthyroid,
wild-type warm mouse



B. Brown fat cell in euthyroid,
wild-type cold mouse



C. Brown fat cell in hypothyroid,
wild-type cold mouse



D. Brown fat cell in
TR-ablated cold mouse

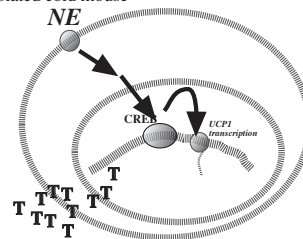


Fig. 3. The repressive action of the thyroid hormone receptor on gene expression. "Warm" and "cold" are figure abbreviations for "close to or at thermoneutral temperature" and "significantly below lower critical temperature." In C, the ball-ended line indicates repression. For further explanation, see text.

Interaction Between Brown Adipose Tissue and Thyroid Thermogenesis

There are both indirect and direct effects of thyroid hormone on brown adipose tissue; the direct effects include the conversion of T_4 to T_3 and the intracellular as well as possible systemic effects of T_3 .

Indirect Regulation of Brown Adipose Tissue Recruitment and Activity by Thyroid Hormone

Irrespective of what the “mechanism” of thyroid thermogenesis is, functionally, in a given situation, hyperthyroid animals will produce more heat than euthyroid—which means that the need for “extra” heat in the cold will be diminished. Many observations (although not all) accordingly support a decreased recruitment of brown adipose tissue in hyperthyroid animals. This effect of thyroid thermogenesis thus does not merit any other explanation than the general control of body temperature. Correspondingly, the brown adipose tissue of hypothyroid animals may display certain characteristics of being hyperrecruited, but as the tissue is unable to produce heat (due to lack of UCP1, see below), the final outcome is more complex (reviewed in Cannon and Nedergaard, 2004).

Brown Adipose Tissue as a Internal and Possibly External T_3 Producer

The circulating form of thyroid hormone is mainly T_4 , and many peripheral tissues possess the ability to convert T_4 to the physiologically active hormone T_3 . In brown adipose tissue, this conversion is performed by type II deiodinase (Silva and Larsen, 1983), the activity of which is increased in the cold due to adrenergically increased synthesis of the enzyme (Jones et al., 1986). This deiodinase undoubtedly provides the brown-fat cells with the necessary T_3 . Earlier calculations have indicated that a significant fraction of circulating T_3 may also have been produced in the brown adipose tissue. However, in mice that lack the deiodinase, the T_3 levels in the serum are not altered (de Jesus et al., 2001; Schneider et al., 2001), indicating that this deiodinase, in brown adipose tissue or elsewhere, is not essential for maintaining normal circulating T_3 levels.

Direct Regulation of Brown Adipose Tissue Recruitment and Activity by Thyroid Hormone

The UCP1 gene has thyroid hormone responsive elements in its promoter. Experiments with thyroid-hormone-receptor-ablated mice have indicated that the (unliganded) thyroid hormone receptor here functions as a repressor (Golozoubova et al., 2004). This leads to the following scenarios, summarized in Fig. 3.

The normal animal in the warmth (A) is euthyroid, i.e., the thyroid hormone receptor (TR) is occupied by T_3 and thus its repressing function is inactivated. However, here in the warmth, the cell receives no adrenergic stimulation and thus no gene expression is induced.

When this animal is exposed to the cold (B), an increased adrenergic stimulation occurs, leading to increased expression of the gene for UCP1.

In animals that are hypothyroid (C), no thyroid hormone binds to the thyroid hormone receptor, which then exerts its repressive action. This means that although the adrenergic signal is (at least) equally strong, the expression of UCP1 is diminished as compared to the case in the euthyroid animal. There are even indications that the adrenergic stimulation may be stronger in the hypothyroid than in the euthyroid animal. There may be two reasons for this. To increase sympathetic activity is apparently a general effect of hypothyroidism (with no known mechanism). More specifically, the adrenergic stimulation of brown adipose tissue could be specifically increased, due to lack of thermoregulatory feedback. Due to the repressed expression of UCP1, the brown-fat cells cannot produce the expected heat, and this may lead to an attempt from the central areas involved in control of body temperature to further increase stimulation.

However, in animals that lack thyroid hormone receptor (D), the repressive function is absent. The expression of UCP1 is therefore, perhaps surprisingly, at least the same as that found in euthyroid animals (Golozoubova et al., 2004). It could be argued that the expression would be expected to be higher—but that would only be the case if euthyroid T_3 levels were not sufficiently high normally to overcome the repressive action. It may be noted that even though there is a higher circulating level of thyroid hormone, this hormone has no receptor to bind to; thus, evidently, UCP1 expression is thyroid hormone-independent in thyroid hormone receptor-ablated animals—but the expression is not lower than that observed in wild-type animals. Thus, only lacking thyroid hormone is much worse than only lacking thyroid hormone receptor.

Why are Hypothyroid Brown Adipocytes Desensitized?

Brown-fat cells from hypothyroid animals, from animals that lack the type II deiodinase (de Jesus et al., 2001), and from thyroid hormone receptor-ablated animals (Golozoubova et al., 2004) all demonstrate adrenergic desensitization. It has been the accepted interpretation of such observations that this indicates that thyroid hormone is important in the adrenergic signalling process. However, all such observations have been made on preparations (cells) “freshly” isolated

from these animals. The adrenergic desensitization observed is akin to the desensitization observed in brown-fat cells isolated from cold-acclimated animals (Nedergaard, 1982), and this means that the response of the cells may be influenced *in vitro* by the environment that the cells had experienced *in situ*: adrenergic hyperstimulation leads to desensitization. Although cells directly isolated from thyroid-hormone-receptor-ablated mice thus are desensitized, brown-fat cells that have been isolated as precursors from such animals and have then developed in cell culture do not show any signs of desensitization (Golozoubova et al., 2004). This demonstrates that thyroid hormone is not necessary for normal function of adrenergic signalling pathways. In extension of this, we propose that the desensitization seen in cells and membranes from hypothyroid animals is a secondary effect of an adrenergic hyperstimulation and not directly related to any function of thyroid hormone in the signalling processes in the brown-fat cells.

Why are Hypothyroid Animals Cold Intolerant?

Hypothyroid animals, as well as thyroid hormone receptor-ablated animals (Golozoubova et al., 2004), do not survive when suddenly exposed to the cold. This cold intolerance is widely understood as being due to a diminished capacity for nonshivering thermogenesis in brown adipose tissue. However, although there is a contribution from any preexisting brown adipose tissue, it is indeed through shivering thermogenesis (i.e., muscle-derived thermogenesis) that mammals acutely exposed to cold initially survive; it takes time to develop a significant capacity for nonshivering thermogenesis in brown adipose tissue. It is therefore more likely that cold intolerance in hypothyroid (and thyroid hormone receptor-ablated) animals is due to muscle problems rather than to brown adipose tissue problems. Indeed, muscle function is impaired in hypothyroid animals (Wiles et al., 1979), as well as in thyroid-hormone-receptor-ablated animals (Johansson et al., 2003).

Nonshivering Thermogenesis and Hibernation

It is evident from the comments above that shivering thermogenesis is the only facultative thermogenesis that is always available, and that nonshivering thermogenesis is really only a practical adaptive replacement. However, it would seem that mammalian hibernation could not have developed without nonshivering thermogenesis, and thus without brown adipose tissue. This is simply because hibernating animals cannot shiver at hibernating temperatures and have to rely on nonshivering thermogenesis for initiation of arousal (during later arousal

they may shiver as well). How mammals ascertain that they possess sufficient nonshivering thermogenesis capacity before entering hibernation remains one of the many wonders of hibernation.

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Alterations in Localization of Hippocampal Protein Kinase C γ (PKC γ), but Not PKC α , - β 1, or - β 2, in European Ground Squirrels During Hibernation

EDDY A. VAN DER ZEE,¹ JENS STIELER,³ ROELOF A. HUT,² MARTIJN DE WILDE,² AND ARJEN M. STRIJKSTRA²

¹ Department of Molecular Neurobiology, University of Groningen, Groningen, The Netherlands.

² Department of Animal Behavior, University of Groningen, Groningen, The Netherlands.

³ Paul Flechsig Institute of Brain Research, Department of Neuroanatomy, University of Leipzig, Germany.

Abstract. Hippocampal signal transduction is largely reduced during the torpid hibernation state. The protein kinase C (PKC) system is a major signal transduction system in the hippocampus. Alterations in the PKC system may contribute to physiological modifications observed during hibernation. We therefore investigated hippocampal Ca²⁺-dependent PKC (PKC α , - β 1, - β 2, and γ) immunoreactivity at various stages of hibernation and continuous euthermia in European ground squirrels (*Spermophilus citellus*). Immunoreactivity of all four PKC isoforms measured in the pyramidal cell layer was significantly reduced during all hibernation stages as compared to euthermia in nonhibernating animals. Thus it is likely that during hibernation PKC-mediated transmembrane signal transduction is reduced. Interestingly, PKC γ immunoreactivity was enhanced during late arousal, whereas the other isoforms did not reveal such an increase during the euthermic arousal period. Further indications for a specific role for PKC γ , in contrast to the other isoforms, was found both in the subcellular distribution and Western blot data. PKC γ immunoreactivity was localized in close association to the nucleus during the torpid hibernation state, and part of PKC γ (but no other isoform) was cleaved into a putatively constitutively active PKM form. These results strongly suggest that PKC γ plays a distinct role in the regulation of (peri)nuclear processes during torpor.

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The Hippocampal PKC System

Protein kinase C (PKC) is abundantly expressed in the hippocampus. PKC is an intracellular messenger system involved in various neuronal signal transduction pathways through phosphorylation of specific substrate proteins by which neurons increase their excitability in response to external inputs (Nishizuka, 1986). To date, at least 12 PKC isoforms have been identified and classified into 3 groups based on their structure and cofactor regulation: classical PKC (cPKC), novel PKC and atypical PKC. The cPKCs consist of four Ca^{2+} -dependent isoforms (α , $\beta 1$, $\beta 2$, and γ) of which only the γ isoform is specific to brain tissue (Nishizuka, 1988; Saito and Shirai, 2002). The ~ 80 kD cPKCs consist of two domains, a regulatory one of ~ 32 kD and a catalytic one of ~ 45 kD connected by a hinge region. PKCs phosphorylate many cellular proteins, catalyzing the transfer of phosphate to certain amino acid residues within proteins. Phosphorylation can alter the folding of the protein and hence their function. This alteration is reversible by subsequent activation of phosphatases removing the phosphate.

The discovery of PKC took place relatively recently in the late 1970s (for a historical review see Van der Zee and Douma, 1997, and references therein). The cPKC isoforms of this kinase are enzymatically fully active in the presence of Ca^{2+} and the phospholipid phosphatidylserine. The kinase is activated in a reversible manner by attachment to membrane phospholipid in the presence of Ca^{2+} . Further analysis showed that a small amount of diacylglycerol (DAG; a minor component of the cellular lipids) significantly increases the affinity of this enzyme for Ca^{2+} and phospholipid. DAG (and inositoltriphosphate) is produced by the hydrolysis of phosphatidylinositol biphosphate (PI turnover). Interestingly, DAG permitted activation of PKC at resting intracellular Ca^{2+} levels. PKC is usually present in an inactive form in the cytosol. As a result of the specific binding of PKC by DAG, which is transiently formed in the membrane, activation of PKC is accompanied by its translocation from the cytosol to the membrane. The duration and magnitude of the DAG signal determines the activation of PKC at the cellular membrane. PKC can be cleaved by proteases like calpain in the regulatory and catalytic domain, and the latter (called PKM) then is constitutively active and no longer Ca^{2+} -regulated (Kishimoto et al., 1989).

Activation of PKC plays an important role in hippocampal synaptic plasticity (both Long Term Potentiation and Long Term Depression; Ramakers et al., 1997). It has been suggested that particularly PKC γ is crucial for synaptic formation and plasticity (Saito and Shirai, 2002). All PKC isoforms are found to

be present in the cytoplasm of the cell body and dendrites. In addition, PKC γ is the only cPKC isoform localized in dendritic spines and the nucleus (Kose et al., 1990; Tanaka and Saito, 1992). This hints at a specific function of PKC γ as compared with the other isoforms.

Hippocampal signal transduction is largely reduced during the torpid hibernation state. The PKC system is a major signal transduction system in the hippocampus. Regulation of hippocampal PKC in hibernating mammals may be crucial for reversible phosphorylation as for example reported for tau protein in European ground squirrels (Arendt et al., 2003) and suppression of metabolic rate (Storey, 1989). The hippocampal PKC system may significantly contribute to physiological (and behavioral) alterations observed during and also after hibernation. Moreover, the hippocampus has been postulated as a brain area involved in regulating the length of torpor bouts, possibly through action of the histaminergic system (Sallmen et al., 2003). The hippocampus displays continuous electrical activity during torpor, in contrast to other brain regions (Strumwasser, 1959).

Experimental Procedure for the Study of Hippocampal PKC in the European Ground Squirrel During Hibernation

We explored cPKC expression in different stages of hibernation, i.e., during euthermic and torpid periods. European ground squirrels (*Spermophilus citellus*; $n = 28$ for immunocytochemistry and $n = 4$ for Western blotting) were used in this study. For origin, housing, and hibernation characteristics of the animals see Hut et al., 2002; Arendt et al., 2003; Strijkstra et al., 2003. Animals were sacrificed in four different stages of hibernation and during continuous euthermia: early torpor (ET; 2.3 [SEM 0.21] days after the onset of the previous [last] arousal to euthermia, $n = 5$), late torpor (LT; 7.1 [SEM 0.1] days, $n = 5$), early arousal (EA; 1.5 [SEM 0.06] hours, $n = 5$), late arousal (LA; 8.3 [SEM 0.05] hours, $n = 5$), and continuously euthermic animals that had ended hibernation (EU, $n = 8$). Arousal was induced (at least 10 weeks after onset of hibernation) by gentle handling at room temperature for three to five minutes. Brain material of nonhibernating animals (EU) was collected six to seven days after cessation of hibernation, initiated by an increase in ambient temperature from 7° C to 25° C in early spring. Body temperature upon perfusion was 36.5 (SEM 0.4)° C. All animals were terminally anesthetized with 2 ml 6% pentobarbital, 10 min before perfusion. Nonperfused fresh brain material of four additional animals was collected for Western blotting. Two hibernating

animals were sacrificed at a similar timing as the LA group (< 7 hours after arousal induction, following > 7 days of torpor) and one hibernating animal as the LT group (> 7 days of torpor). The fourth animal was sacrificed during continuous euthermia in summer. For immunocytochemistry, animals were perfused with 4% paraformaldehyde. Immunostaining (on coronal brain sections of 25 μm) and/or Western blotting was performed for PKC α (two rabbit polyclonal antibodies were used: C20 [Santa Cruz] and P4334 [Sigma]); PKC β 1 (C16 [Santa Cruz]); PKC β 2 (C18 [Santa Cruz]; and PKC γ (two antibodies were used: C19 [Santa Cruz], and 36G9 [produced in our own lab; see Cazaubon et al., 1989 for origin]. All antibodies were raised against the catalytic domain of PKC with the exception of 36G9, which was raised against the regulatory domain of PKC γ .

Hippocampal PKC Immunoreactivity During Hibernation in the European Ground Squirrel

Western blots of EU animal hippocampus samples stained for the four cPKC isoforms (using the Santa Cruz antibodies) revealed prominent bands at ~ 80 kD, which corresponds to PKC kD values of other species (Van der Zee et al., 1997a). No additional immunopositive kD values were observed, indicating that EU animals had no constitutively active hippocampal PKC (see for PKC γ Fig. 2B).

PKC immunoreactivity for all antibodies used was found in European ground squirrel hippocampus. Both PKC α antibodies and both PKC γ antibodies showed similar results for the respective PKC isoform. Fig. 1 shows CA1 pyramidal cells stained for PKC γ (see figure legend for more details on staining patterns). The observed alterations in PKC γ localization in the different stages of hibernation are PKC γ -specific; for the other cPKC isoforms immunostaining was found along the cell membrane and in the cytoplasm corresponding to the picture seen for PKC γ in LA and EU animals. It should be noted that the (peri)nuclear localization of PKC γ in ET, LT, and EA animals was also present in most other brain regions, with the exception of the hippocampal granule cells of the dentate gyrus.

Optical density measures in the CA1 pyramidal cell layer are shown in Fig. 2. Compared to euthermic animals in spring, PKC immunoreactivity was significantly reduced for all four cPKC isoforms ($p < 0.05$), indicating a general reduction of PKC-mediated hippocampal signal transduction. In the course of hibernation, no fluctuations were found for PKC α , β 1, or β 2. In contrast, PKC γ immunoreactivity increased significantly during the euthermic phase (compare

EA and LA; $p < 0.05$), and the staining intensity of LA animals was significantly higher compared to that in animals of the late torpor phase (LT; $p < 0.05$). This finding suggests isoform-specific regulation of PKC γ . The difference in cellular localization between EA and LA animals may in part cause the difference in OD values, but it cannot be excluded that *de novo* synthesis of PKC γ takes place or that the enhanced OD for PKC γ somehow reflects changed (enhanced) activity levels of PKC γ (as has indeed been shown for these two antibodies in relation to learning and memory; Van der Zee et al., 1997b).

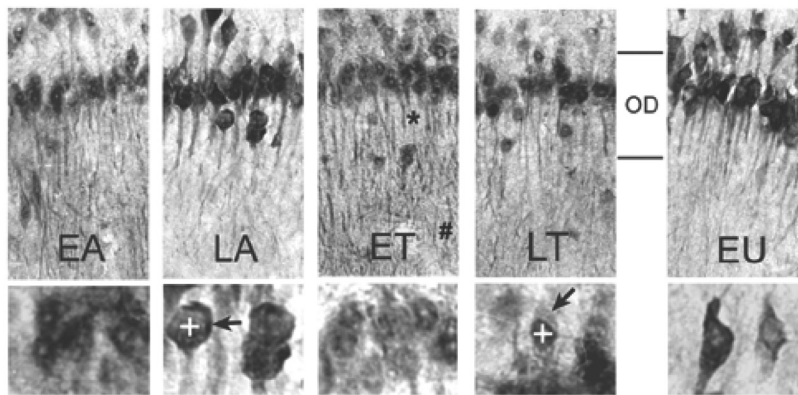


Fig. 1. PKC γ -immunoreactivity in CA1 pyramidal cells of the dorsal hippocampus of European ground squirrels in different stages of hibernation (upper panels). Below these photomicrographs enlarged images of individual cell somata are shown (lower panels). Nucleus and cell membrane are indicated in the LA and LT lower panels by + and arrow, respectively. Proximal and distal parts of apical dendrites are indicated in the ET upper panel by * and #, respectively. The optical density (OD) of PKC γ immunoreactivity (and that of the other cPKCs) was measured in the pyramidal cell layer including the most proximal part of the apical dendrites, as delineated in the upper row (see Fig. 2). In EU animals during spring, PKC γ staining is dense in cytoplasm and associated to the cell membrane. In addition, the proximal part of the apical dendrites are clearly stained. During hibernation, the subcellular localization of PKC γ differs. PKC γ is more abundantly present in the distal part of the apical dendrite and concentrated around the nucleus during EA, ET, and LT. As a consequence, in these animals individual pyramidal cells are more difficult to discern. In contrast, LA resembles the picture of that of EU animals in spring.

The Western blots for the four cPKC isoforms revealed no apparent changes between the different stages of hibernation, with the exception of PKC γ . An additional band of ~ 40 kD was found in hibernating animals (LA and LT; see Fig. 2B for this band in an LT animal as compared to an EU animal). This band was confirmed with a third PKC γ antibody (PKC66; Zymed), suggesting that PKC γ was partly cleaved into PKM, the constitutively active form (Kishimoto et al., 1989). The (peri)nuclear staining for PKC γ , however, cannot be explained by this form of PKC γ , because the ~ 40 kD band is present in LA animals while PKC γ localization is no longer (peri)nuclear. Moreover, both PKC γ antibodies (C19 and 36G9; binding to the catalytic and regulatory domain, respectively) reveal the (peri)nuclear localization of PKC γ , which strongly suggests that at

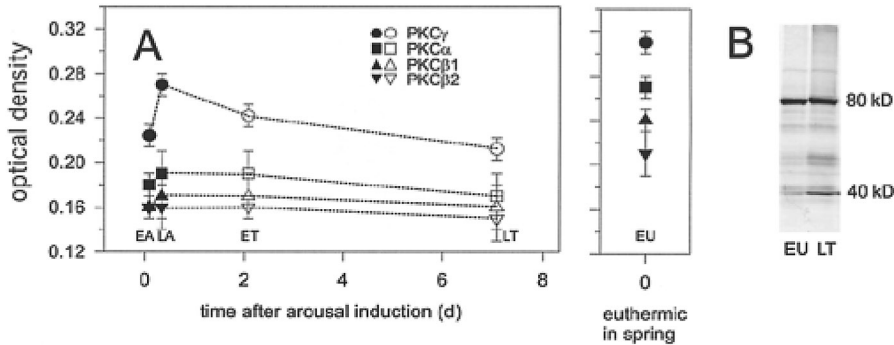


Fig. 2. (A) Optical density of PKC immunoreactivity in the CA1 pyramidal cell layer of hibernating European ground squirrels as a function of time since arousal induction (left panel), or continuously euthermic animals in spring (right panel). Solid symbols indicate euthermic situations, and open symbols indicate torpid situations ($n = 5$ for each situation during hibernation and $n = 8$ for continuously euthermic spring animals). During hibernation, a cyclic pattern in PKC γ immunoreactivity occurs, but not in immunoreactivity of PKC α , $\beta 1$, or $\beta 2$. PKC γ immunoreactivity is reduced during torpor as indicated by a negative association from LA to LT (Spearman rank correlation coefficient $r_s = -0.75$; $p < 0.01$). For all cPKC isoforms, immunoreactivity is higher in continuously euthermic spring animals as compared to animals in any stage of hibernation. (B) Western blot of hippocampus samples stained for PKC γ (C19) in a euthermic summer animal (EU) and a hibernating animal sacrificed after seven days of torpor (LT). In the LT animal an additional band at ~ 40 kD was present.

least the majority of the (peri)nuclear staining represents the intact PKC γ molecule. It is currently unknown whether the (peri)nuclear localization is due to anchoring of PKC γ to the nuclear membrane (and/or endoplasmic reticulum) via specific anchoring proteins or by modulated interactions of PKC γ with phospholipids.

How Kinase-Specific are the Observed Alterations for PKC γ ?

Obviously, within the cPKC family only PKC γ showed characteristic adaptations to hibernation as described above. One may wonder, however, whether other kinases regulating synaptic plasticity and putatively linked to histaminergic signal transduction behave more similar to PKC γ or to the other cPKC isoforms. To begin to address this issue, immunostaining was performed for the cAMP-dependent protein kinase A (PKA) and the phosphorylated form of PKA (pPKA) as well as for calcium/calmodulin-dependent kinase type II (CaMKII) and the phosphorylated form of CaMKII (pCaMKII). These results show that neither of these two prominent hippocampal kinase systems underwent the striking subcellular alterations during the course of hibernation as seen for PKC γ , although pCaMKII was dramatically enhanced in the cytoplasm of pyramidal cells of the LT group (preliminary data not shown). This further indicates that PKC γ plays a distinct role in the hibernating brain, of which the function needs to be clarified in future experiments.

Speculations on Functional Implications

The changes in subcellular localization suggest that PKC γ (but not the other isoforms) present in the cell body is selectively redirected (either actively or passively) from the cell membrane to a (peri)nuclear site at the beginning of torpor. This relocation is reversed upon arousal, although delayed since EA animals that are euthermic still have their PKC γ localized around the nucleus. Clearly, PKC γ in torpid neurons acts on different substrates and serves a different regulatory function (most likely related to (peri)nuclear processes) as compared to PKC γ in LA or continuously euthermic neurons. Dendritic PKC γ , however, is not redirected to the nucleus. PKC γ -mediated transmembrane signal transduction at dendritic spines during hibernation therefore remains intact (or is even enhanced), whereas at the cell body level a reduction of PKC γ -mediated transmembrane signal transduction is implicated due to the fact that PKC γ is unavailable for this function. It is presently unknown whether this redirected PKC γ is involved in the induction and/or maintenance of the torpid state, or that it

serves a neuroprotective role in anticipation to arousal. PKC γ is considered to be one of the stress signaling molecules in the bat brain during arousal from hibernation to protect neurons when the brain undergoes high oxygen demands (Lee et al., 2002). Interestingly, PKC γ is the sole form of cPKC present in the hibernating bat brain (Mehrani and Storey, 1997).

In conclusion, since the brain specific PKC γ shows torpor specific patterns of action, this kinase may have an important role in brain regulatory aspects of ground squirrel hibernation. PKC γ could be involved in regulation of (peri)nuclear processes during torpor while being involved in synaptic remodeling in dendritic spines during arousal. Furthermore, it is tempting to speculate that this dendritic PKC γ is important for the continuous hippocampal electrical activity during hibernation (Strumwasser, 1959), since part of the histaminergic signal transduction is mediated by PI turnover (Sallmen et al., 2003), activating PKC.

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The Role of the Medial Septum in the Control of Hibernation

IRINA YU. POPOVA AND YURI M. KOKOZ

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow region, Russia

Abstract. The ability of the brain of hibernators to maintain functional control during hibernation at very low body/brain temperatures has not yet been explained. It is known that the preopticohypothalamic area controls thermoreception and body temperature in hibernators (South et al., 1972; Heller, 1979; Ruby et al., 1998). At the same time, it was suggested that the septohippocampal system is important in the organization of periodic and urgent arousals during hibernation (Shtark, 1970; Beckman and Satinoff, 1972; Heller, 1979). It was proposed that the medial septum (MS) acts as a “sentry post” that triggers the activation of the forebrain and, as a consequence, the arousal of the hibernating animal (Belousov, 1993). This viewpoint is confirmed by the paradoxically high responsiveness of MS neurons to neuropeptides during hibernation (Pakhotin et al., 1993; Zenchenko et al., 2000). It was shown that the high responsiveness of MS during hibernation is formed by the influence of extraseptal structures (Popova et al., 2003a), the strongest effect being exerted on pacemaker MS neurons responsible for the hippocampal theta-rhythm. This influence of extraseptal structures (which is probably mediated through compounds of peptide nature) is strongly pronounced during hibernation and severely limited in summer.

Introduction

Hibernation, like any other complex adaptive behavior, is controlled by brain structures. Limbic brain structures play an important role in the control of the waking–hibernation cycle (Chatfield and Lyman, 1954; Strumwasser, 1959;

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

Shtark, 1970; South et al., 1972; Heller, 1979; Beckman and Stanton, 1982). According to the hypothesis of Heller (1979), the entry into hibernation begins with a change in the threshold of hypothalamus temperature and a reduction in the excitatory influence of the reticular formation (RF) of the brain stem (Fig. 1A). This leads to an increase in the descending inhibitory effect of the hippocampus on RF, which further reduces the activity of RF. The maintenance of hibernation is accomplished by the tonic inhibitory influence of the hippocampus on RF and hypothalamus. An important role in this model was assigned to the generator of the hippocampal theta-rhythm, the medial septum (MS), whose activation leads to a reduction of the inhibitory influence of the hippocampus on RF, which may trigger the transition to the waking state. However, further studies of Heller and some other authors showed that the key role in the organization and maintenance of hibernation is played by the thermoregulatory and circadian mechanisms of the hypothalamus (Beckman and Satinoff, 1972; Wunnenberg et al., 1978; Heller et al., 1989; Belyavski and Fedorova, 1999).

Another model of the control of the central nervous system over the waking—hibernation cycle suggested by Beckman and Stanton (1982) is based on the assumption that the hippocampus and RF have a mutual inhibitory effect on each other (Fig. 1B). According to this model, the hippocampus contains an inhibitory network, which rhythmically oscillates from a high to a low level of activity and vice versa, with a period corresponding to the duration of a hibernation bout. The entry of the animal into hibernation is associated with the enhancement of the hippocampal inhibitory influence on RF (it reduces its excitability and suppresses a flux of signals crossing RF) and the hypothalamus (it reduces the functional level of the physiological systems of the organism). During the hibernation bout, the inhibitory influence is progressively reduced, and the excitability of RF increases, leading to the suppression of the hippocampal inhibition and finally to the arousal of the animal.

A third scheme of the interaction of limbic structures in the brain of hibernators was proposed by Belousov et al. (Fig. 1C); (1990, 1993). Emphasis in this scheme was placed on MS and its relationships with the hippocampus, lateral septum, and hypothalamus. According to this model, the arousal from hibernation is realized by the following mechanism: the appearance or the enhancement of signals (circadian, circannual, and sensory) entering the MS gives rise to the theta-rhythm in the hippocampus; during the theta-activity, the descending signals of the hippocampus, while travelling through the lateral septum, affect the hypothalamic mechanisms that regulate the level of metabolism and body

temperature, arousing the animal. It was suggested that the MS of the hibernator brain acts as a “sentry post” that responds to significant sensory stimuli (Belousov, 1993). This hypothesis was based on comparative studies of MS neuronal activity in brain slices from hibernating and summer waking ground squirrels, which showed that MS neurons from the brain of hibernating animals (but not neurons of the structures adjacent to the MS) had a paradoxically high discharge frequency and responsiveness to the electrical stimulation of afferents (Belousov et al., 1990; Pakhotin et al., 1993). The authors suggested that the high level of excitation of septal neurons during hibernation is maintained by compounds of endogenous nature (specific hormones, neuropeptides, and neurotransmitters). In view of this, several endogenous biologically active compounds (neuropeptides TRH, TSKYR, TSKY, DY, and neurotransmitters noradrenaline and serotonin), which are supposedly involved in the control of winter hibernation, were tested on MS neurons from brain slices of hibernating animals (Belousov, 1993; Zenchenko et al., 2000). It was found that MS neurons of sleeping animals have a paradoxically high responsiveness to neuropeptides compared with MS neurons of summer waking animals, whereas the responsiveness to neurotransmitters did not depend on season. As opposed to MS, neurons of the other brain structures (lateral septum, medial preoptical area, and hippocampus) from the brain of both waking and hibernating animals were not sensitive to these peptides (Belousov, 1993; Popova, 2002).

Season-dependent changes in the responsiveness of MS neurons can be explained either by intraseptal changes or by the influence of extraseptal structures (first of all, hypothalamus and RF). To determine which of the mechanisms of regulation of neuronal activity is realized, we analyzed the contribution of afferents from extraseptal structures to the seasonal excitability of MS neurons (Popova et al., 2003a, 2003b). For this purpose, a procedure of chronic basal undercutting of the MS area in winter hibernating animals was developed, which completely eliminated the afferents to MS, the direct contact with the preoptical area, and the descending influence of the septohippocampal system on the hypothalamus and stem structures. The activity of neurons was studied on slices from the brain of three groups of ground squirrels *Citellus undulatus*: summer wake, winter hibernating, and winter hibernating with chronic undercutting of MS. The neuropeptides TSKYR, TSKY, and DY and the monoamines serotonin and noradrenaline were tested.

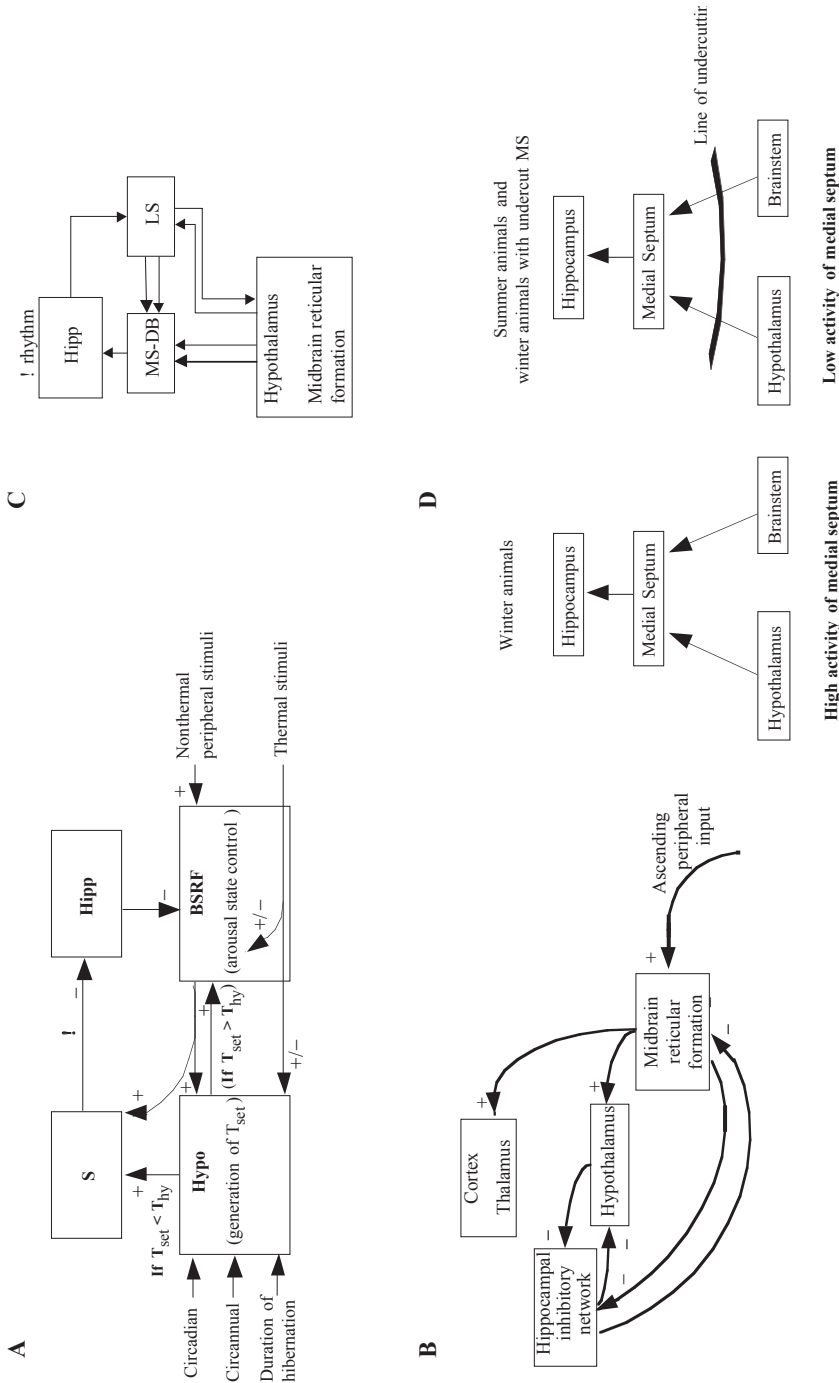


Fig. 1. Models of the involvement of different brain structures in the control of hibernation.

- A. A model of how the brain stem reticular formation (BSRF), the hypothalamus (Hypo), and the limbic system as represented by the septum (S) and the hippocampus (Hipp) might interact to control the daily sleep/wakefulness cycle and the hibernation/lethermia cycle proposed by Heller (1979). Excitatory influences are indicated by “+” and inhibitory influences are indicated by “-”. T_{set} refers to the hypothalamic temperature threshold for the metabolic heat-production response, and represents the activity of theta-rhythm pacemaker cells in the septum. The inputs to the hypothalamic compartment represent influences on the generation of T_{set} .*
- B. Hypothetical model of hippocampal-brainstem control of central activity state during hibernation proposed by Beckman and Stanton (1982). “-” inhibitory influences; “+” exciting influences.*
- C. The supposed model participating of the septal area in neural control of hibernation/lethermia proposed by Pakhotin et al. (1993). MS-DB, medial septal complex (medial septal nucleus of diagonal band of Broca); LS, lateral septal nucleus; Hipp, hippocampus; mFB, medial forebrain bundle. The thick arrows are the neural pathways of another mediators nature.*
- D. A model of the formation of specific properties of the medial septum during hibernation by the action of peptides from extraseptal structures.*

Results and Discussion

Spontaneous Activity of MS

The analysis of experimental data indicated that the high reactivity of MS to the neuropeptides during hibernation is due to the tonic influence of the extraseptal structures: after chronic basal undercutting, the parameters of the MS neuronal activity of hibernating ground squirrels become identical to those of summer waking animals (Fig. 2). However, the effect of (neurotransmitters) noradrenaline and serotonin was completely independent of the state of animals and the intactness of afferents. Consequently, it is hardly probable that these monoamines are involved in the control of specific seasonal excitability of septal cells.

Evoked Activity of MS

All neuropeptides tested effectively modulated the responses of neurons to electrical stimulation in all three groups of animals. However, a comparison of hibernating and active animals revealed a marked tendency for the “seasonal” effect of peptides. For instance, TSKYR substantially lengthened the duration of inhibition during hibernation, whereas in summer-active ground squirrels the inhibition was either shortened or blocked (Zenchenko et al., 2000). In the group of hibernating animals with the undercut MS, the peptides effectively modulated the evoked responses; however, the analysis revealed no distinct tendency in changes of responses to the peptides. Based on these data, it can be stated that the specificity of the action of neuropeptides on the evoked responses of MS neurons in animals being in different functional states is provided by septal afferents.

Spontaneous and Evoked Activity of MS Neurons

A comparison of the effects of the peptides on the spontaneous and evoked activity of septal neurons gave an interesting result. It was found that the spontaneous and evoked activities of neurons responsive to the peptides change independently of each other (Popova et al., 2003b). Based on this finding, it was assumed that neuropeptides affect MS neurons in two ways: they change the level of spontaneous activity through the direct modulation of the pacemaker potential of cells and regulate the responses to electrical stimulation by either pre- or postsynaptic action. To verify this hypothesis, we compared the activity of neurons in normal and calcium-free media (Mg^{2+} ions were substituted for Ca^{2+} in an equimolar ratio) using a standard method. The data obtained in this series of experiments showed that these peptides modulate the activity of

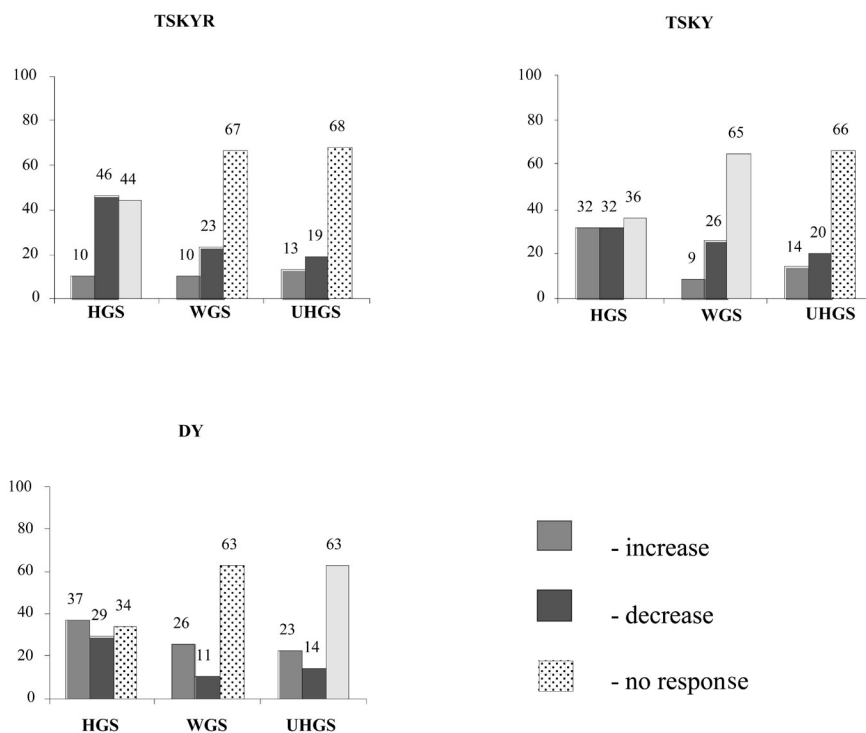


Fig. 2. Histograms of the distribution of changes in the MS neuronal spontaneous activity in three groups of animals under the influence of neuropeptides. The groups of experimental animals (HGS, hibernating ground squirrels; WGS, wakening ground squirrels; and UHGS, hibernating ground squirrels with the undercut septum) are indicated below the graphs. The effects of neuropeptides TSKYR (neokyotorphin), TSKY, and DY are shown. The number above the column indicates the percentage of neurons with the corresponding changes in spontaneous activity (increase and decrease in discharge frequency and no response) under the influence of the peptides. The number of neurons is given in percent; the total number of neurons in a group is taken as 100%.

neurons having different types of spontaneous activity by different ways. The effect of neuropeptides on neurons having a regular pacemaker-like and irregular activity is mainly mediated via the synaptic transmission, and the effect on the activity of burst cells is accomplished through either the synaptic pathway or the direct modulation of the mechanism of endogenous generation of charges.

It should be noted that the disconnection of the septohippocampal system from the hypothalamic and brain stem structures does not disturb the ability of the animal to enter into hibernation and maintain this state by the action of temperature and seasonal factors. This means that the descending influences of the septohippocampal system are indeed not necessary for the entry into hibernation and the tonic support of this state. These results do not fit into the hypothesis of Beckman and Stanton (1982), according to which the chronic undercutting of connections should disturb the hibernation rhythms. At the same time, our data agree well with the data of Heller et al. (1989) on the primary role of the hypothalamic area in the hibernation control and develop the idea of Belousov (1993) about the specific role of the medial septum in the waking-hibernation cycle.

The above data suggest that the increase in the reactivity of MS is due to the seasonal influence of extraseptal structures (Fig. 1D). This influence (which is probably mediated through compounds of peptide nature) is markedly pronounced during hibernation and severely limited in summer. The data reported in the literature (Kobayashi, 1989; Yu and Cai, 1993; Cui et al., 1996; Nurnberger et al., 1997) suggest that the most probable source of tonic specific influence on MS during hibernation is the hypothalamus, which is responsible for the regulation of temperature, circadian rhythms, and metabolism. Owing to the high excitability during hibernation, MS may become sensitive to sensory stimuli, which enables the septo-hippocampal system to filter incoming signals and arouse the brain in the case of emergency.

Acknowledgements

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Proteolysis in Hibernators

FRANK VAN BREUKELLEN

Department of Biological Sciences, University of Nevada, Las Vegas, Nevada, USA

Abstract. A key feature of mammalian hibernation is depressed protein synthesis. Normal cellular metabolism involves the regulated degradation of proteins. In the absence of significant protein synthesis, protein degradation would quickly deplete the cell of protein pools. Ubiquitin-dependent proteolysis is responsible for the degradation of most regulatory proteins. During torpor, ubiquitylated proteins increase. However, proteolytic processing by the 26S proteasome is markedly reduced at temperatures typical of torpor. Hibernators appear to effectively utilize the reduced body temperatures of torpor to depress protein degradation.

Introduction

In response to environmental stress, many mammals employ a state of torpor or hibernation wherein core body temperatures (T_b) below 0° C have been reported (for reviews see Carey et al., 2003; van Breukelen and Martin, 2002). However, throughout the course of a typical six month hibernation season, one-to-three-week-long bouts of torpor are interrupted by brief (approximately 20–24 h) periods of euthermia. Concordant with reduced metabolism, protein synthesis is depressed during torpor but resumes during the interbout arousal. Radiolabeled amino acid incorporation during hibernation falls to 0.13–0.5% of active euthermic rates (Zhegunov et al. 1988). Much of this depression appears to be the result of an acute block of translational initiation when T_b reaches 18° C as the animal enters torpor (van Breukelen and Martin, 2001). Thus, hibernators appear to effectively utilize the reduced body temperatures associated with torpor to depress the energetically consumptive process of protein synthesis. The inability to translate proteins at low T_b means that the hibernator must restrict

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its translational efforts to the interbout arousal with its inherent warmer T_b . Presumably, this “part-time” translational effort is sufficient to supply required protein products to maintain cellular integrity and viability throughout both torpid and aroused states. However, the availability of protein products during torpor is governed not only by the synthesis of these products but also by their regulated degradation. In light of the negligible protein synthesis associated with torpor, one must ask what happens to protein degradation? Without new protein synthesis, continued proteolysis as a function of normal cellular metabolism would quickly result in the net depletion of key cellular proteins. Based on a half life of 20–30 h for a relatively stable protein (e.g., Rogers et al., 1986; Dice, 1987) and negligible new synthesis, a protein would be depleted to 20% of steady state levels in only 45–70 h if degradation rates remained constant. Yet torpor bouts are usually from one to three weeks in length. This disparity suggests a marked reduction in proteolysis during hibernation.

Ubiquitin-dependent proteolysis is responsible for the degradation of most short-lived or regulatory proteins (Ciechanover et al., 1984; Rock et al., 1994). Ubiquitin-dependent proteolysis consists of two essential steps: (1) the marking of a target protein with ubiquitin, a very highly conserved 76-amino acid polypeptide, and (2) subsequent recognition and degradation of that marked target protein. Proteins that are conjugated to a polymer of ubiquitin moieties are signaled for degradation by the 26S proteasome whereas monoubiquitylated proteins are generally targeted for endocytosis and subsequent proteolysis in the lysosome (for reviews, see Mykles, 1998; Lecker et al, 1999; Pickart, 2001).

Ubiquitin conjugates increased as much as two to three fold during torpor (Fig. 1; van Breukelen and Carey, 2002). Typically, higher levels of ubiquitylated proteins correlate with increased rates of proteolysis (e.g., van Breukelen and Hand, 2000; Munro and Pelham, 1985). An alternative and perhaps more parsimonious explanation for the increase in ubiquitylated proteins is that proteolysis was downregulated by passive temperature more so than ubiquitylation. Thus, ubiquitylated proteins accumulate during torpor.

The model for a net accumulation of ubiquitin conjugates during torpor predicts that proteolysis but not ubiquitylation per se is markedly reduced at the low temperatures typical of torpor. Cleavage of the fluorogenic substrate, Suc-LY-AMC, by the 26S proteasome as a function of assay temperature supports this hypothesis (Fig. 2; Qureshi and van Breukelen, unpublished). Proteolytic activity is highest in a lysate derived from an LT animal when assay temperature is 37° C. At assay temperatures of 0–5° C (similar to T_b during

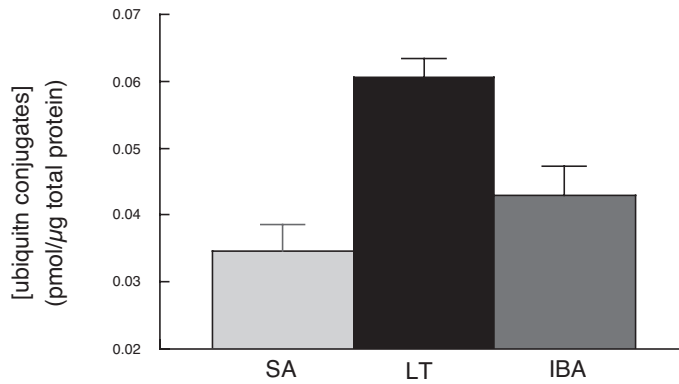


Fig. 1. Ubiquitin conjugate concentrations in the liver of golden-mantled ground squirrels. Squirrels were collected during summer (SA), late in the torpor bout (LT), and during the interbout arousal (IBA). Values represent means \pm SE, $n = 3$. LT was statistically different from IBA or SA (ANOVA; $p < 0.05$). Redrawn from van Breukelen and Carey (2002).

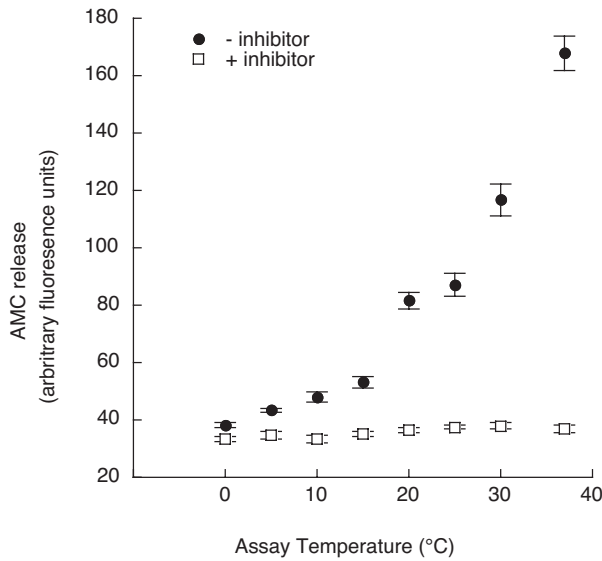


Fig. 2. Effects of temperature on 26S proteasome activity. A lysate was made from an LT animal. The fluorogenic substrate Suc-LY-AMC was added and the release of AMC was monitored (closed symbols). The reaction was inhibited by the presence of MG115, a specific blocker of the 26S proteasome (open symbols). Values represent means \pm SE, $n = 3$.

hibernation), fluorescence activity is reduced to near background levels (similar to values obtained using a lysate with the highly specific proteasome inhibitor, MG115). In other systems, a marked temperature dependence for proteolysis has been noted as well (see van Breukelen and Carey, 2002, for review). Hibernators appear to effectively utilize the reduced body temperatures of torpor to reduce protein synthesis and protein degradation. The use of the interbout arousal allows for a resumption of protein metabolism. Thus, hibernators apparently rely on low body temperatures during torpor to afford protection from proteolysis.

In summary, the physiological constraints of torpor limit protein synthesis, and continued degradation of proteins during torpor would jeopardize the ability of the hibernator to return to the euthermic state. Hibernators apparently downregulate ubiquitin-mediated proteolysis in a rather unconventional way; ubiquitin conjugates remain high throughout the torpor bout but the low body temperatures in the torpid squirrel severely reduce protein degradation. As the animals rewarm as part of the periodic interbout arousal, the turnover of proteins and replenishment of protein pools resumes.

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Post-genomic Approaches to the Mechanisms of Cold Response in Fish and Hibernating Small Mammals

DARYL WILLIAMS,¹ L. ELAINE EPPERSON,² ANDREW R. COSSINS,¹ JANE FRASER,¹
WEIZHONG LI,¹ SANDRA MARTIN,² AND ANDREW Y. GRACEY³

¹ School of Biological Sciences, University of Liverpool, Liverpool, UK

² University of Colorado School of Medicine, Denver, CO, USA

³ Hopkins Marine Laboratory, Stanford University, Pacific Groves, CA, USA

Abstract. We have adopted an intensive microarray-based transcript screening strategy to explore the responses to cold exposure of two contrasting species, both of which experience seasonal cold, namely the common carp, *Cyprinus carpio* and the golden mantled ground squirrel, *Spermophilus lateralis*. For both we have developed a panel of sequence-characterised cDNA probes and printed these on poly-lysine coated glass slides giving ~15K and ~10K probes, respectively. Analysis of tissue cDNA has revealed a large number of cold-regulated transcripts in carp. This comprises ~260 transcripts that are coordinately regulated in all seven tissues examined and 23 clusters of 1,700 transcripts that are regulated in a tissue-specific manner. Gene ontology profiling reveals rich patterns in the biological processes under regulation and provides a new and highly integrative overview of the system-wide response. By contrast, preliminary work on the ground squirrel liver shows a very limited regulation of genes between animals in hibernation and during brief arousal periods but substantial differences between these winter animals and summer awake animals. These contrasting responses match differences in the overall cold-response strategy of the two species, namely sustaining biological activity despite cold in the carp and suppressing biological activity during winter periods in the ground squirrel.

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Introduction

Extreme cold arguably represents the most profound environmental challenge for living organisms. The large and variable reduction in biological rate processes causes dislocation to all manner of otherwise integrated cellular processes, which can lead to events that ultimately cause tissue damage and even death (Cossins and Bowler, 1987). Organisms experiencing periodic cold usually display a suite of adaptations, ranging from the behavioural and energetic to the metabolic and enzymatic (Somero and Hochachka, 2002). Current views of the process of adaptation have been built up over the past 100 years by the conventional form of hypothesis testing. However, while this has given insights into a range of mechanisms at all levels of organisation, in most cases of interest to the comparative biologist the level of understanding is often less than deep, particularly at the molecular and genetic levels. This can be attributed to a general lack of genetic and even experimental tractability in the most commonly studied animal models, at least relative to that enjoyed by the most widely used so-called genomic “model” species such as yeast, *Drosophila*, and mouse. It is thus not uncommon to translate a research problem onto one of the more tractable species, often to good effect.

However, contemporary post-genomic techniques now offer alternatives to the abandonment of environmental models, namely the use of transcript or protein screening on the global or near-global scale. Whilst they have been developed for use in sequenced model species they can be adapted for nonsequenced models of environmental significance (Gracey and Cossins, 2003). These permit the investigation of transcript or protein abundance without limiting the number of targets, and this enables the simultaneous examination of thousands or tens of thousands of entities in one experiment. This new capability opens up open-ended screening approaches that do not involve the adoption of a specific *a priori* hypothesis. This so-called “discovery” approach thus enables exploration and discovery of responses and of novel genes without limit, which then can be subjected to further analysis by conventional hypothesis testing (Gracey et al., 2001). Importantly, it also provides a system-wide overview of the response rather than one restricted to just a candidate gene or two.

We have adopted the transcript screening approach to provide overviews of cold-adaptive physiology in two contrasting animal models, namely a cold-adapted poikilotherm, the common carp, and a hypothermic homeotherm, the golden mantled ground squirrel. Both species suffer from the same technical

problem, a lack of genomic resources and infrastructure, and production of these is a necessary prerequisite for progress.

Technology Hurdles to Microarray Analysis in Nonmodel Species

Broad transcript screening can be undertaken by two contemporary techniques, namely SAGE (Velculescu et al., 1995) and microarray technology (Schena et al., 1995). The latter has proved to be adaptable particularly for species lacking sequence resource (Gracey and Cossins, 2003). The microarray consists of a series of DNA probes, of known identity, spotted in regular patterns onto a two-dimensional surface, each hybridising with a specific fluorescence-labelled target cDNA from the fluid phase (Eisen and Brown, 1999). Each probe is capable of discriminating a different target simply on the basis of its base pairing specificity. There are two alternative types of array based on the source of probe, namely oligonucleotide and cDNA (Schena et al., 1998). Whilst the former requires extensive sequence knowledge to predict and then synthesise reliable complementary probes for each gene, the latter can be produced directly by cloning the cDNA into a suitable vector, and PCR amplification of the insert. At the outset, production of probe can occur in the absence of knowledge of the sequence identity, a feature that makes it the technique of choice for species of most interest to comparative physiologists working in comparatively small research communities on species characterised by a lack of sequence resources. In reality, the production of cDNA libraries produces clones with unequal representation, such that any random collection would contain highly skewed mixtures. This has led to the use of normalisation, a procedure that equalises the representation of common and rare transcripts (Carninci et al., 2000), and subtraction, which enriches those transcripts that vary in abundance between two physiological, disease or experimental conditions (Diatchenko et al., 1996).

cDNA collections require identification by nucleotide sequencing, and the collection of sequences need to be clustered into groups that reflect individual genes. These lists are presented as searchable databases in which genes are furnished with a series of annotation terms that relate to biological function at the level of process or the molecule. These are crucial in the final interpretation of expression profiles of possibly large numbers of genes. The resulting amplified inserts are then spotted onto poly-L-lysine coated glass slides using a spotting robot and the arrays hybridised to labelled target (cDNA) by overnight incubation. The resulting pattern of fluorescent spots is quantitatively analysed and

evaluated statistically across a possibly large number of arrays, each one offering an independent value for each probe (Eisen et al., 1998)

Analysis of Cold Responses in the Common Carp

We have conducted a large-scale analysis of the transcriptional response of a teleost fish, the common carp, *Cyprinus carpio*, to environmental cold. Given that cold has a direct physical effect on every molecule, cell, and organ of the animal, we anticipated that the response to cold would be profound. A microarray-based approach currently offers the best route to addressing the full complexity of the integrated responses.

The first step was to establish a collection of cDNA clones that would serve as a source of microarray probes. We prepared 14 cDNA libraries using mRNA isolated from seven carp tissues that had been exposed to all relevant stressors (cold, heat, or hypoxia, sampled over the full time-course). This yielded a very diverse mRNA population that contained a substantial proportion of the transcripts of interest. All libraries were normalized and some underwent a subtraction step in which cDNAs already isolated were physically removed from the library, thereby maximising representation and reducing redundancy on the microarray. To assess the quality of each library, a small subset of clones, normally 48–96 cDNAs per library, were sequenced from their 5' end, and the proportion of full-length clones and the rate at which new genes were discovered was determined. Based on these criteria, between 400 and 2000 cDNA clones were picked at random from each library, their inserts amplified by PCR, and the amplicons robotically arrayed, giving a total of 13,440 mostly nonredundant cDNAs. Of these ~10,000 clones were 5' end-sequenced and subjected to BLAST-homology searching. A fully annotated database (*carpBASE*) containing gene sequences and identities was constructed using an in-house informatics package (EST-Ferret). Both can be viewed at <<http://legr.liv.ac.uk>>.

We used this microarray for a time-course analysis of transcript expression in seven tissues, including liver, brain and heart, of carp subjected to graded cooling regimes (from 30° C and held at either 23, 17, or 10° C), and compared them with fish maintained throughout at the control 30° C temperature. At each time-point, five fish were sampled and tissues archived at –80° C. Changes in tissue mRNA levels were determined by competitive hybridization to carp microarrays, each RNA sample being compared to a common “reference” mRNA by hybridisation to two arrays with fluor-reversal. In total, 177 separate

RNA samples were hybridised to 354 microarrays to investigate the differential gene expression associated with cooling.

One indication of the reliability of our screen for cold-regulated genes was the identification of a $\Delta 9$ -stearoyl-CoA desaturase gene. We have previously highlighted the functional significance of desaturase expression in carp liver tissue (Tiku et al., 1996; Trueman et al., 2000). Here, the microarray reveals that desaturase expression increases upon cooling in all of the seven tissues we examined, not just liver as previously reported. Fig. 1 illustrates a general observation that transcript expression was a graded function of the thermal perturbation and was transient, for example, peaking on days two and five of the 17° C and 10° C cooling trajectories, respectively, in the case of the brain. We have identified a number of other potentially important genes that show powerful transcript induction in the cold in all tissues, including a cold-inducible RNA-binding protein, and a number (>10) of less well-characterised but cold inducible genes that have been implicated in human disease.

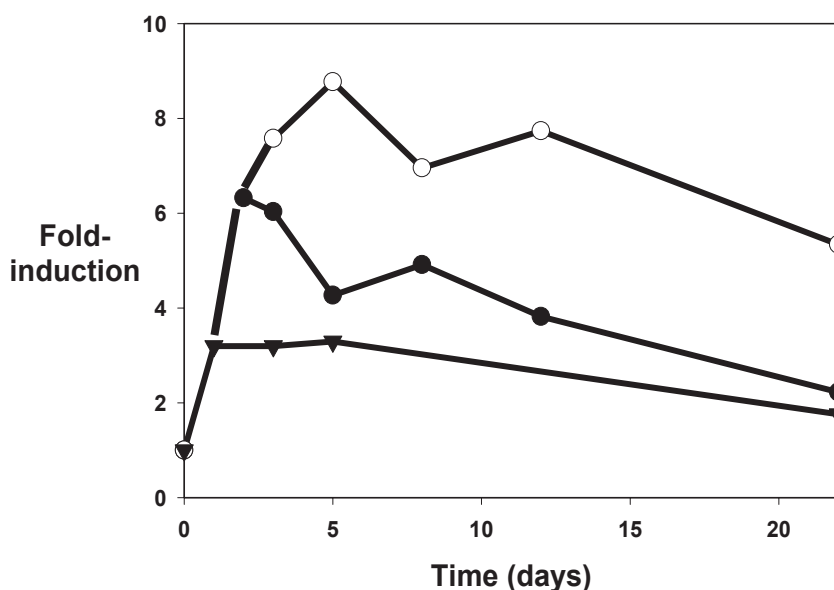


Fig. 1. Time dependence of desaturase transcript expression in brain of common carp subjected to cooling. This data was determined from large-scale microarray screening experiment on ~130 specimens exposed to progressive reductions of temperature from 30° C to the indicated temperatures.

Extensive statistical processing of the global data set has revealed a “common” transcriptional program that is induced in all cold-exposed tissues. In total, we found that ~260 unique cDNAs were significantly differentially expressed in all seven tissues. The majority (>96%) increased in expression upon cooling, indicating that the common response is dominated by a strong coordinated induction of genes. This disproportionate response may reflect a basic paradigm of cold-acclimation: that poikilotherms compensate for the rate-depressing effects of cold by synthesising more enzymes in order to increase biochemical performance. To interpret the physiological role of the common response, we classified the genes according to their function and biological process using their gene ontology (GO) annotations (Ashburner et al., 2000). Of the 180 annotated genes, 40 were associated with transcription and translation, 37 with solute transporters, 35 with protein catabolism, 21 with cell stress, and 18 with intermediary metabolism.

We have also identified ~1,700 additional genes that cluster into 23 groups showing significant responses in one or more tissues. This provides a coherent overview of the tissue specificity of cold-responsive genes. GO profiling with adoption of multiple sampling correction highlights the biologically significant features of each cluster. For example, in the cluster characterised by upregulated genes in intestinal mucosa, the GO transport category is up-represented, a feature that has long been recognised as important to intestinal cold-adaptation. Using this method we have identified substantial changes to carbohydrate and lipid metabolism in the principal core tissues.

Transcript Responses to Hibernation in a Small Mammal

We have also undertaken an analysis of transcript profiles following hibernation in the golden mantled ground squirrel, *Spermophilus lateralis*, and to a lesser extent in the thirteen-lined ground squirrel. Again it was necessary to produce a set of sequence characterised cDNA clones, and we collected ~10,000 cDNA probes from heart, liver, brain, and embryos and used this to probe transcript levels in tissues of radio-telemetered squirrels with precisely known thermal histories and thermal status at the time of death. *SquirrelBASE* contains a current build of the annotated sequence database created by *EST-Ferret* (again at <<http://legr.liv.ac.uk>>)

Initial experiments have been directed at liver in which for *S. lateralis* we compared five specimens from each of three groups, namely summer awake (SA), winter interbout aroused (WA), and deep hibernation (DH), using

ANOVA to identify genes that showed significant changes in expression between the physiological states. A preliminary analysis of these data indicates that while the transcript profile of SA group was substantially different to the two winter groups, there were few differences between WA and DH groups. Analysis of tissues samples from thirteen-lined ground squirrel using the *S. lateralis* array revealed an essentially identical picture, the two species being sufficiently similar in DNA sequence to allow cross-hybridisation.

Of the few genes that were different between WA and DH, the most obvious were those involved in urea cycle such as carbamoyl-phosphate synthase, arginosuccinate synthase, arginase, and ornithine transporter 1, all of which were repressed during deep hibernation. A disproportionate number of P450 genes also seem to be repressed. Together these changes are consistent with a general decline in the liver detoxification function. A second notable feature is the increased variation in transcript profiles between individuals in the SA group compared to either of the winter groups. We are progressing with the analysis of other tissues.

Conclusions

These two experiments demonstrate the power of model-independent screening approaches to illuminate the mechanisms underpinning a complex phenotypic response of the entire organism. In carp we have defined common elements to cold-responses of different tissues and also tissue-specific responses that fits particular tissues to the cold. The technique not only offers broad overviews of the entire system during the transition from one state to another, but also allows the identification of novel genes or genes whose role was previously unappreciated. Moreover, the relative magnitude of gene responses can be assessed within any one tissue and between tissues, giving insights into the most important and most consistent aspects of the response. This knowledge can suggest new hypotheses regarding detailed mechanism of response and thereby provide direction to future research.

Preliminary evidence suggests that the changes evident on cooling of carp were substantially larger and more diverse from those observed in the ground squirrel, at least for liver. We found very few differences at the level of the transcript between squirrels in deep hibernation or aroused from hibernation, but there are substantial differences between these animals and those in the summer. This does not mean that there would not be differences in protein complement nor in the activity of expressed proteins, but it does suggest that regulation of

transcription is not an important means of adjustment between the hibernating and aroused states during deep winter. This distinction between the carp and the ground squirrel relates to the different energetic strategies being deployed; carp maintains level of activity as temperature is reduced, at least down to 8–10° C, while the ground squirrel depresses activity during hibernation as part of an energetic saving strategy. One seeks to function in the cold while the other merely survives the cold until clement conditions return.

An important issue in the functional interpretation of genomic data is the relative importance of responses at other levels of regulated expression and the way these relate to protein function. While we offer transcript data for key genes with well-demonstrated activity responses, there remains a question mark over the functional significance of large-scale transcript data. However, the existence of clear-cut and large-scale patterns of response in both of our experiments, many of which have plausible and internally consistent interpretations, argue that transcript regulation is indeed an important element in the overall process of physiological regulation. Indeed, the observations made form the basis for formulating more direct and detailed hypotheses, for example, in the importance of novel genes or of shifts in metabolism. Well-developed transcript data also provides a robust means of classifying the phenotype, allowing separation of specimens into groups that relate to their status.

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Use of Suppression Subtractive Hybridization to Elucidate Novel Gene Products Related to Physiological Events in a Hibernator

GREGORY L. FLORANT,¹ CHRIS PITTMAN,¹ AND SCOTT A. SUMMERS²

¹ Department of Biology, Colorado State University, Fort Collins, CO 80523, USA

² Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO 80523, USA

Abstract. We used the relatively new and highly effective technique of suppression subtractive hybridization (SSH) to selectively amplify cDNA fragments that are differentially expressed in marmot (*Marmot flaviventris*) white adipose tissue (WAT) during the summer and winter period. By making a winter-specific cDNA library, we were able to isolate clones of gene fragments that are up-regulated in winter. Thus far we have analyzed 12 of the 1,600 clones that were identified, and Northern blot analysis confirmed that these clones were all up-regulated or solely expressed in winter. Interestingly, a BLAST search of several clone sequences suggested that these 12 clones represent immunoglobulin genes. This result was confirmed by the presence of T and B cells identified in some of the same fat tissue using immunohistochemistry. With many more clones to sequence, we are hopeful that in the future we may find clones that are related to changes in food intake and lipid metabolism.

Introduction

Many species of mammalian hibernators survive the winter by metabolizing lipids within white adipose tissue (WAT) that were previously deposited during the prior summer and early fall. The marmot (*M. flaviventris*) is a large hibernator that nearly doubles its body mass during the summer in anticipation of winter when food supplies are virtually nonexistent (Florant, 1998). This increase in

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body mass is primarily in the form of lipid (i.e. triacylglycerol) that has the inherent advantage of being high in energy and therefore is a preferred fuel source during periods of fasting. Thus, it is not surprising that WAT is the primary tissue type that increases in mass during the weight gain period prior to fasting. In addition to its high energy content, WAT also has recently been shown to synthesize and secrete several novel proteins that appear to be important in maintaining energy homeostasis. Two such proteins are leptin and adiponectin. Leptin binds to receptors in the hypothalamus that are specifically involved in food intake and energy metabolism, and adiponectin acts in the several tissues, including muscle and liver, affecting fatty acid oxidation and hepatic glucose production, respectively (Havel, 2002). In addition, leptin appears to be positively correlated with increasing adiposity, while adiponectin is negatively correlated with fat mass (Unger, 2003). In light of these findings, we proposed that WAT might produce and secrete additional physiologically active molecules specific to the summer (feeding/lipogenic) period and/or molecules specific to the winter (fasting/lipolytic) period. In order to assess whether our hypothesis is true, we used suppression subtractive hybridization (SSH) to subtract out gene sequences that occur in both states in order to elucidate novel gene sequences that are up regulated or unique to either the winter or the summer condition. As such, the resultant winter-specific proteins would be different from those produced in summer. If so, then perhaps we could identify novel proteins that are involved in the preparation for winter (i.e., long-term fasting). This would provide us with important information about how marmots are able to switch from a lipogenic to a completely lipolytic state.

Materials and Methods

Animals and Tissue

All adipose tissue was taken from adult yellow-bellied marmots trapped in Colorado and housed at Colorado State University. Colorado State University's Animal Care and Use Committee have approved all protocols involving these animals.

RNA Isolation

Total RNA was isolated using a phenol-based method (TOTALLY RNA kit, Ambion). Briefly, frozen tissue was ground in a mortar and pestle and mechanically homogenized in denaturation solution. The homogenate was centrifuged briefly, separated from the lipid layer, and exposed to two rounds of organic

extraction. Total RNA was then precipitated in the presence of a carrier. mRNA was subsequently purified from total RNA using an Oligo-dT based method (Oligotex mRNA kit, Qiagen). Two rounds of Oligo-dT selection were performed to maximize removal of rRNA and any residual DNA.

Suppression Subtractive Hybridization (SSH)

SSH was conducted on winter and summer WAT from one animal (#1102) using Clontech's PCR-Select cDNA Subtraction kit. This kit is a modified version of a procedure previously published (Diatchenko, 1996). In brief, 0.5 µg of WAT mRNA from each season was used as starting material. Winter cDNA was the target of our investigation and therefore, became the tester cDNA population through ligation of two types of adaptors. The adaptors are short oligos that contain two primer-binding sites, one that is shared between the two types of adaptors and one that is different. The products of the secondary PCR reaction should be gene segments that are up regulated or solely expressed in winter. Analysis of these gene segments was conducted using Clontech's PCR-Select Differential screening kit. The products of the second PCR reaction were T/A cloned into pGEM-T Easy (Promega) and transformed into electrocompetent DH5α in order to create the subtracted winter library. Approximately 1,600 clones were picked. Two identical cDNA arrays were made from the first 96 clones by blotting 2 µL of a PCR reaction of each clone onto two Hybond-N+ (Amersham Biosciences) membranes. The arrays were hybridized with different ³²P probes. One probe was made from the pool of subtracted winter gene segments (the same sequences used to make the library), and the other probe was made from the pool of subtracted summer gene segments (subtraction had also been done in the opposite direction—summer tester/winter driver). Both arrays were visualized using a phosphorimager screen, Storm Imager, and ImageQuant software (Molecular Dynamics). Clones that were positive on the winter screened cDNA array and negative on the summer screened cDNA array hopefully represent truly differentially expressed sequences. Twelve clones have been sequenced, and a BLAST search against NCBI's mammalian database was completed.

Northern Blotting

Ten µg of total RNA per sample were run on 1% denaturing agarose gels and transferred to Hybond-N+ membranes (Amersham Biosciences) using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad). The membranes were then

probed with three different clones. Random labeled ^{32}P probes were made using the Ladderman Labeling Kit (TAKARA Bio Inc.) Blots were visualized using Kodak BioMax MR film, and densitometry measurements were acquired using ImageQuant software (Molecular Dynamics).

Pathology

Summer fat samples from animal #302 and winter fat tissue samples from animals #302 and #1102 were analyzed by H&E staining and immunohistochemistry to detect the presence of lymphoid tissue.

Results

High quality mRNA was isolated from WAT biopsies obtained in winter (January) and summer (July) (Fig. 1). Once the mRNA was isolated, cDNA fragments from each season were produced and the opposing season's sequences were subtracted. An internal control sample confirmed that we had effective subtraction (data not shown), and Fig. 2 illustrates that we had equal loading of RNA on our gels. Fig. 3 illustrates PCR amplified winter-specific clones and the accompanying differential screening. Based on this array, the majority of our nearly 1600 clones were either winter-unregulated or expressed solely in winter.

We next tested whether these clones were seasonally regulated in other animals. Northern blot analysis using clone 1P1E7 indicated that this clone was also winter up-regulated in other animals (Fig. 4). It was clearly up-regulated in animal #1102, and it was up-regulated to a significant degree in two of the three animals tested (#1201 and #201) and slightly up-regulated in a third (#302). In addition, we performed northern analysis on these animals using two additional clones and similarly found that these clones were also up-regulated in winter (Fig. 5). The three clones that we isolated were not from the same mRNA sequence as far as we were able to determine.

In order to determine the genes most homologous to several of our clones, we conducted a BLAST search of NCBI's mammalian database. Our results suggest that the twelve clones we tested are most homologous to immunoglobulin genes (Table 1).

The results of the immunohistochemistry indicated that T cells were present in the summer fat sample of animal 302 and winter fat sample of animal 1102; however, only B cells were detected in winter fat tissue of animal 302.

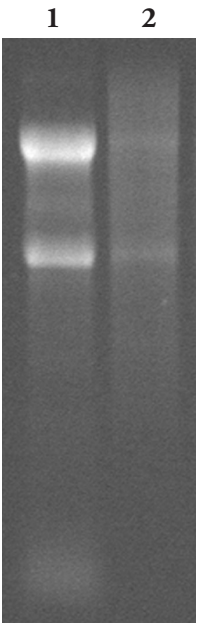
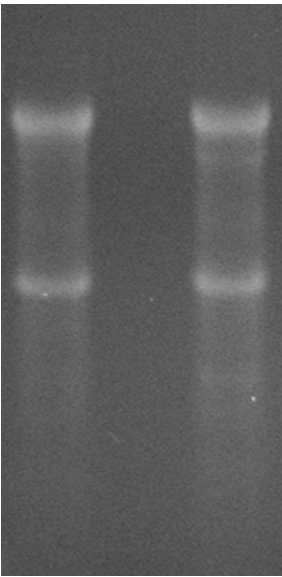
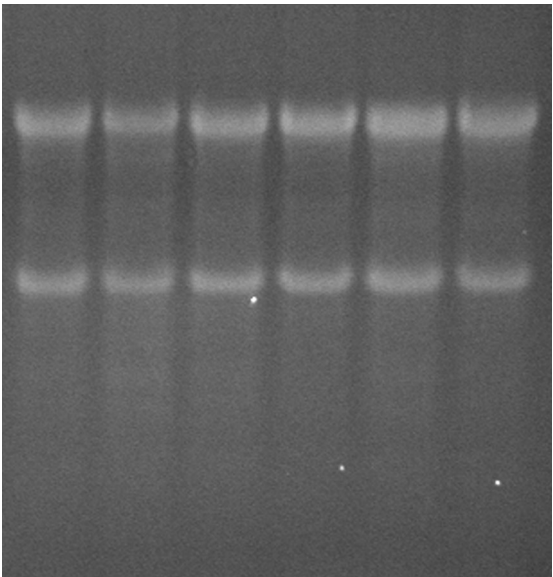


Fig. 1: Ethidium bromide stained 1% denaturing agarose gel of total RNA (lane 1) and mRNA (lane 2) isolated from marmot #1102 WAT.



(A)



(B)

Fig. 2. EtBr stained agarose gels of total RNA illustrate equal loading. (A) animal #1102: lane 1, winter (W), and lane 3, summer (S). (B) lanes 1 and 2: #1201 W and S; lanes 3 and 4: #302 W and S; lanes 5 and 6: #201 W and S.

(A) PCR amplified clones

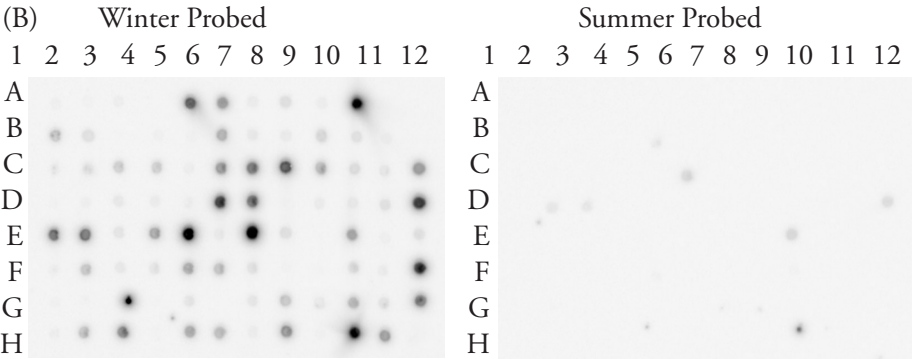
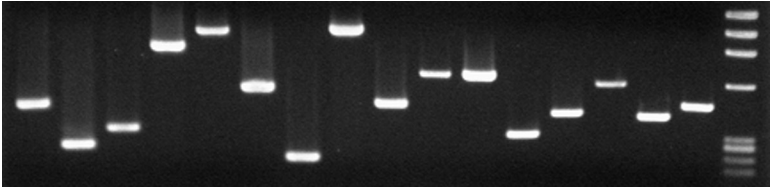


Fig. 3. (A) Representative EtBr stained 2% agarose gel of several PCR amplified clones from the winter library. (B) Differential screening of two identical cDNA arrays made from 96 clones from the winter library. One array (left) was screened with the pool of subtracted winter sequences, and the other array (right) was screened with a pool of subtracted summer sequences.

Discussion

Differential gene expression between winter (hibernation) and summer states in hibernators has been investigated previously (reviewed in Carey et al. 2003). For example, prior studies indicate that alpha2-macroglobulin is up regulated in ground squirrel liver during winter. This protein is a protease inhibitor that plays a role in regulating blood clotting. The clotting times of hibernators are known to be greatly increased in the winter, presumably improving microcirculation (Epperson and Martin, 2002.). In addition, the mRNA for c-fos, junB, and c-jun are differentially expressed in the brain of ground squirrels (O'Hara et al., 1999). These studies, however, did not use WAT and the technique used is unlikely to differentiate rare mRNA transcripts. Unlike these prior studies, we are applying a SSH technique that allows one to equalize transcript abundance

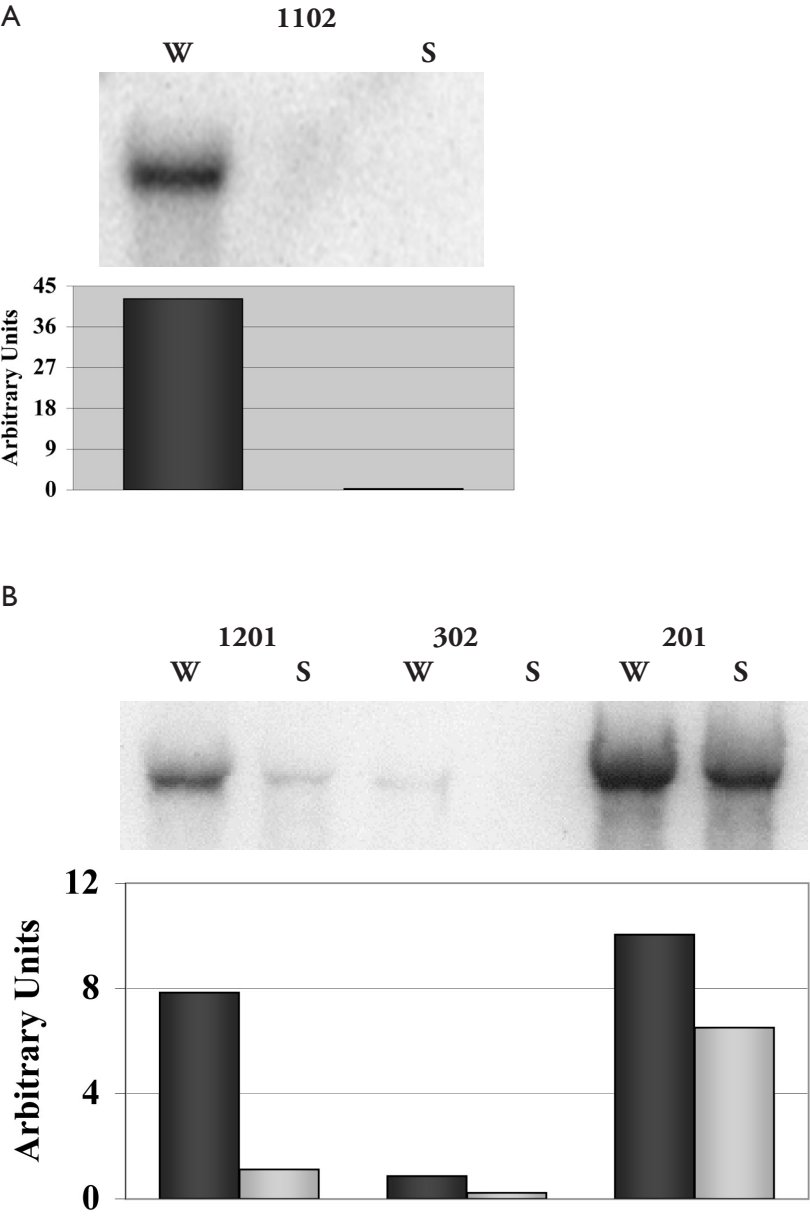


Fig. 4. (A) Northern blot of WAT total RNA from animal #1102 demonstrates clear winter up-regulation of clone 1P1E7. (B) Northern blot of WAT total RNA from three additional animals (1201, 302, 201) also demonstrates winter up-regulation of clone 1P1E7. (W = winter, S = summer.)

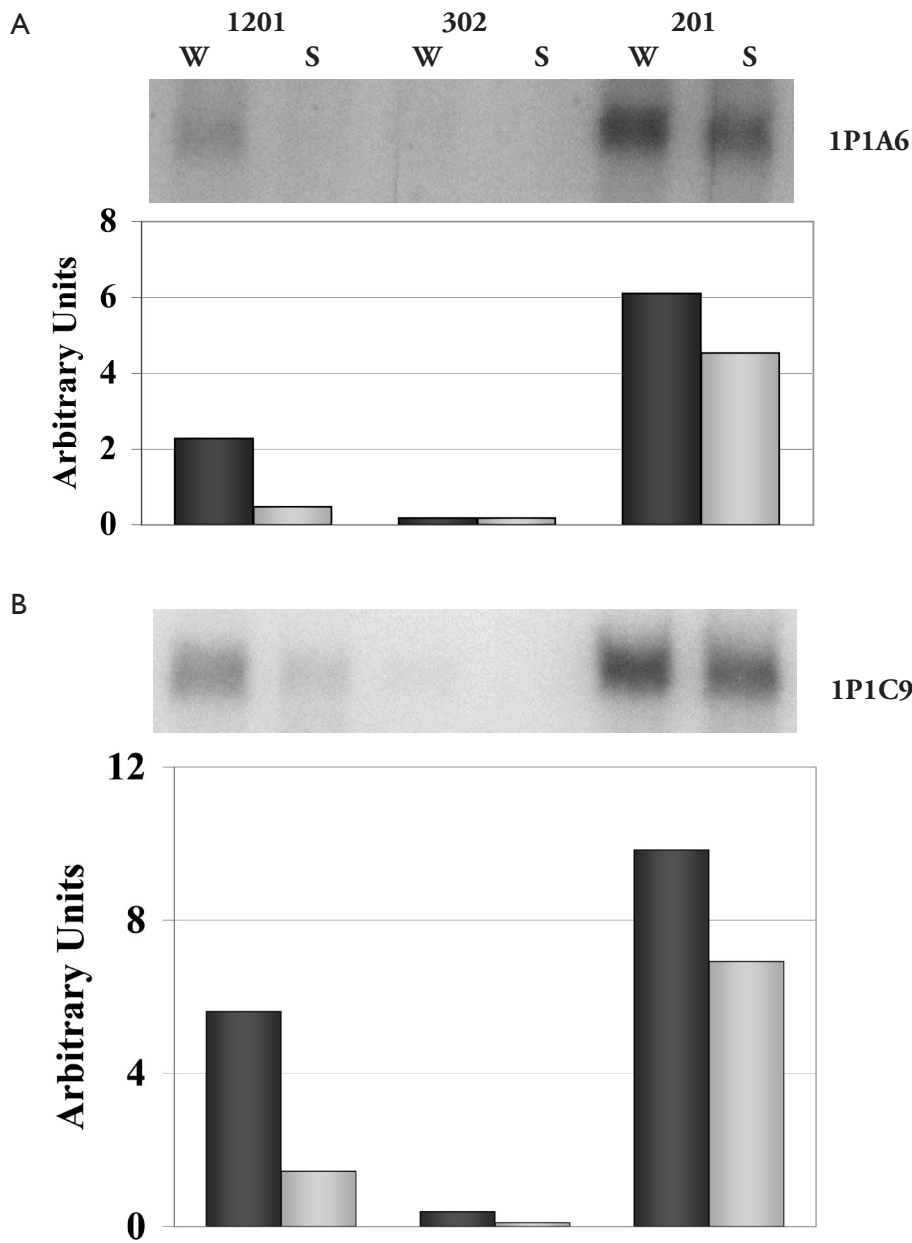


Fig. 5. Northern blots of WAT total RNA from the same three animals (1201, 302, 201) used in the last figure, using two additional clones as probes (1P1A6, 1P1C9), demonstrate the same trend of winter up-regulation.

Table 1: Comparison of the twelve clones with the results from the NCBI BLAST search. There was high homology with mammalian immuno-globulin genes.

Clone Name	Length	Accession # of best hit	Start/Stop Coordinates	Subject Start/Stop Coordinates	nt Identical/nt Aligned	Identification
1P1A5	156	RABIGLCD	1 / 156	41 / 196	131 / 156	Rabbit Ig germline lambda-chain C-region
1P1A6	556	AB064140.1	204 / 472	380 / 112	226 / 269	<i>Homo sapiens</i> IGK mRNA for Ig kappa light chain VLJ region
1P1A10	380	AK093806.1	29 / 350	867 / 1188	276 / 322	cDNA FLJ36487, highly similar to <i>Homo sapiens</i> mRNA for Ig lambda heavy chain
1P1C8	381	AK093806.1	32 / 353	867 / 1188	275 / 322	cDNA FLJ36487, highly similar to <i>Homo sapiens</i> mRNA for Ig lambda heavy chain
1P1C9	423	AB064225.1	189 / 340	362 / 512	129 / 152	<i>Homo sapiens</i> IGL mRNA for Ig lambda light chain VLJ region
1P1D6	364	AF045537	7 / 296	710 / 999	242 / 291	<i>Macaca mulatta</i> Mamu IgG-rh1 heavy chain mRNA
1P1D12	417	AB064235.1	1 / 350	176 / 525	300 / 350	<i>Homo sapiens</i> IGL mRNA for Ig lambda light chain VLJ region
1P1E7	380	AK093806.1	29 / 350	867 / 1188	276 / 322	cDNA FLJ36487, highly similar to <i>Homo sapiens</i> mRNA for Ig lambda heavy chain
1P1F5	414	AB064164.1	68 / 383	210 / 525	268 / 316	<i>Homo sapiens</i> IGL mRNA for Ig lambda light chain VLJ region
1P1F12	412	RRI GG2C	89 / 301	780 / 992	173 / 213	<i>R. rattus</i> mRNA for Ig heavy chain constant region
1P1H2	356	AF124178	91 / 354	106 / 366	223 / 264	Clone PBAL3, <i>Homo sapiens</i> lambda 1 Ig light chain variable region mRNA
1P1H10	380	AK093806.1	31 / 352	867 / 1188	276 / 322	cDNA FLJ36487, highly similar to <i>Homo sapiens</i> mRNA for Ig lambda heavy chain

and subtract differentially expressed genes in the same procedure, dramatically increasing the probability of obtaining rare transcripts. As shown herein, this approach effectively identified genes that were differentially expressed in winter as opposed to summer. However, the genes identified are unlikely to be solely WAT specific genes. Thus, we believe that the sensitivity of the technique led to the identification of genes that were differentially expressed in the sample, but which could originate from contaminating cell types.

That immune cells could have contaminated our mRNA was confirmed by the immunohistochemistry results, which suggested the presence of lymphoid tissue in all of the fat samples that we investigated. However, this conclusion is only based on tissues from two animals, and clearly more evidence is needed to confirm any difference in immune function or status between seasons. Still, it is interesting that the only gene fragments that were up-regulated in winter were immunoglobulin genes because it has been suggested that immune function may be altered between the hibernation state (i.e., low tissue temperature) and after the animal has aroused (Prendergast et al., 2002). As we were concentrating on finding genes especially up-regulated in fat cells, our finding that all twelve of our up-regulated cDNAs isolates were immunoglobulin genes was somewhat surprising. Whether there is a difference between immune function during the summer and winter is unclear but certainly is worthy of investigation.

In future, to be certain that only white adipose cell mRNA is being used in the SSH technique, we have modified our isolation procedure of WAT cells. By first isolating the adipose cells from the fat pad (e.g., using floatation after digestion), we can be more confident that the mRNA isolated from the tissue truly originates from fat cells and not other contaminating cells. Indeed, the fact that the twelve clones we tested are homologous to immunoglobulin genes suggests that our initial cell samples were contaminated with other cell types, including immune cells. In addition, our findings could suggest that animals have increased vascularization of the fat depot during the winter.

We believe that this technique, once fully developed, will uncover potentially new proteins that are involved in the physiological changes required to undergo successful hibernation. In addition, we hope to uncover gene sequences that are important for and involved in the regulation of food intake and the metabolic switch from lipogenesis to lipolysis.

Acknowledgements

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Clinical Applications and Limitations of Hypothermia

PHILIP E. BICKLER

Department of Anesthesia, University of California at San Francisco, San Francisco, CA

Abstract: Experimental studies suggest that mild to moderate hypothermia is a potent means of protecting tissues, especially the brain, from ischemic injuries. Two recent trials demonstrated that hypothermia after cardiac arrest improves survival and neurological outcome, a major milestone. However, the clinical application of hypothermia has been limited in other arenas by a lack of outcome studies and failures of clinical trials in head injury and in cerebral aneurysm surgery. Further clinical benefits depend on increased understanding of the protective effects of hypothermia at the cellular and molecular levels and increased clarity as to the processes that limit the cold tolerance of mammalian tissues.

Introduction: The Clinical Problem of Ischemic Diseases

Protecting tissues from ischemic damage is important in a variety of clinical settings. Stroke is a major cause of death and permanent disability, striking over 300,000 Americans each year. The only therapies proven to improve stroke outcome are those that involve reversing the arterial blockage. Chemical neuroprotection or hypothermia have not been proven effective, despite about 200 clinical stroke trials (American Heart Association Stroke trials website: www.strokecenter.org/trials). Although moderate or profound hypothermia is a mainstay in open-heart surgery, repairs of the aorta, and coronary artery bypass grafting, these procedures have up to a 30% incidence of post-operative neurocognitive impairment and up to 9% incidence of stroke (Ahonen and Salmenpera, 2004). Hypothermia is also a critical component of the procedures used for storage and transport of organs for transplantation. Yet, even for kidneys, the hardest of the major organs, cold storage time is limited;

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transplantation success decreases markedly after 24 hours of preservation. I believe that one conclusion from these examples is that, even with hypothermia, we still lack treatment for the very common ischemic diseases that cost our society lives, lost productivity, and resources. Improving the benefits of hypothermia, by decreasing hypothermia-related complications, has the potential of partially changing this situation. In this review, I will focus primarily on protecting the brain because it is the most ischemia-sensitive organ and thus far has proved to be the most difficult to protect.

Mechanisms of Ischemic CNS Injuries and the Impact of Hypothermia

CNS injuries caused by trauma, hypoxia, or ischemia are a diverse group of diseases. The injury cascade in each of these disorders is very complex and presents a myriad of targets for potential intervention. Briefly, the major groups of events that result in acute or delayed cell loss and dysfunction during and after brain ischemia include energy failure, excitotoxicity, calcium dysregulation, free-radical injury, inflammation, and apoptosis (Lo et al., 2003). A failure of neurogenesis to repair the injured brain probably also occurs. Brain ischemia is a chronic disease, with the evolution or maturation of the injury occurring over many days to weeks. Recently, the immediate post-ischemia period has come into focus as a particularly critical period for intervention, as suggested by both laboratory and clinical studies with post-ischemia application of free-radical scavengers, anti-inflammatory drugs, and hypothermia (Zausinger et al., 2003). As discussed below, results from clinical trials show that hypothermia is beneficial even when the initiation of hypothermia is delayed several hours after the ischemic insult.

Hypothermia is the most consistently successful way to protect neurons in experimental ischemia ever studied. Further, it may be of unique value in decreasing the process of injury that is delayed from the ischemia period itself. It provides benefit in global ischemia (cardiac arrest), focal ischemia (stroke models), and before, during, or after ischemia. Hypothermia works as a neuroprotective strategy in many species of animals, including rodents, rabbits, dogs, and primates.

Beginning about 15 years ago, a resurgence of interest in the neuroprotective effects of hypothermia occurred. In rodents, brain temperature during intra-ischemic period was found to be a strong determinant of the extent of cerebral infarction (Busto et al., 1992). A major correlation was found between the

protection afforded by hypothermia and a reduction in the amount of excitatory neurotransmitters released by the ischemic brain (Busto et al., 1989). The protective effects of even small reductions in brain temperature greatly increased awareness of the importance of temperature in determining ischemia outcome and gave enhanced recognition to the fact that ischemic injury transcended energy failure. From 1989 on, scientists studying brain ischemia had to control brain temperature very closely, lest hypothermia produced by the neuroprotective agent itself confound the outcome. Of particular interest is the fact that *post* ischemia hypothermia provides a benefit in this and other animal models. The mechanisms of hypothermia's protection include reduction in free radicals, inflammatory mediators (Deng et al., 2003), and reduced elaboration of apoptotic signaling molecules by preserving mitochondrial integrity (Yenari et al., 2002). The biological effects of hypothermia on the cascade of ischemic injury therefore are biologically plausible.

Clinical Applications of Hypothermia: Positive and Negative Trials

One of the most prominent uses of hypothermia is to facilitate arrest of the heart during cardiac surgery. Cardiac surgery with cardiopulmonary bypass is associated with a predictable incidence of CNS dysfunction that accounts for significant morbidity and mortality. Embolic events, alterations in cerebral blood flow, hypoperfusion of other organs, reperfusion injury, and inflammation are probably all contributory to the injury. The degree of hypothermia typically varies with the type of surgery. Hypothermia used for deep hypothermic circulatory arrest for repair of the aorta or giant aneurysms in the brain is usually 15–18° C in adults and lower in children. In valve replacement surgery, the patient's temperature is usually 25–32° C and in coronary artery bypass grafting (CABG) the temperature is often 28–30° C but is sometimes as high as 33–35° C. These temperatures are higher than those used 10 to 20 years ago, reflecting shorter bypass runs facilitated by newer surgical approaches. It might be supposed that objective data exists to support the use of hypothermia for heart surgery. Studies have produced conflicting results, and good randomized trials have not been done for procedures involving deep hypothermia. The best data is for CABG surgery. CABG patients randomized to a brain temperature of 28–30° C had the same rate of cognitive deficits as those randomized to be at 35.5–36.5° C during bypass (Grigore et al., 2001). A meta-analysis of 19 trials examining possible benefits of hypothermia yielded a similar conclusion (Rees et al., 2001). However, some data showing protective benefits do exist. Slower rewarming of

slightly hypothermic patients reduces the incidence of postoperative neurocognitive decline (Grigore et al., 2002), and hyperthermia is decidedly negative in terms of outcome (Grocott et al., 2002). Data on the benefit of deeper degrees of hypothermia for circulatory arrest are anecdotal. Randomized, prospective clinical trials comparing hypothermia to normothermia for these dangerous procedures are unlikely to be approved by a medical ethics committee.

A very positive development has been the publication of two studies showing that mild hypothermia benefits survivors of cardiac arrest. This work (Bernard et al., 2002; Hypothermia Study Group, 2002) is already recognized as a watershed. Perhaps most remarkably, the benefit involved demonstration of a positive benefit on survival when hypothermia was instituted after the cardiac arrest. In the Bernard et al. study, randomization to the hypothermia group was associated with a reduction in mortality from 74% to 51%.

In contrast, hypothermia has failed in a 1,000-patient study of neurologic deficits after surgery for brain aneurysm repair (Michael Todd, Univ. Iowa, pers. comm.). This type of aneurysm surgery is associated with an incidence of postoperative deficits of about 15%. Similarly, hypothermia for treatment of head injury has failed to be proven to benefit patients (Clifton et al., 2001), although the hypothermia was sometimes delayed in its application. Furthermore, the patients in the hypothermia group had more hospital days with complications than did those in the normothermia group. It is unclear as to why so much laboratory and early clinical data struck such an optimistic note in favor of hypothermia, other than to suggest that the mean time from injury to hypothermia of eight hours in the head injury trials missed a window of therapeutic opportunity.

Limitations of Hypothermia

While it might be supposed that hypothermia is a very low risk intervention, this is not necessarily true. While mild hypothermia presents minimal risks (Hindman et al., 1999), it is still far from clear that the benefits always outweigh the risks. All clinical hypothermia trials must include a control group of normothermic patients with the express purpose of examining the negative side effects of hypothermia. In this section, I will discuss some of the factors that limit the clinical application of hypothermia and why this approach is necessary.

Cooling and rewarming patients is not always easy. Vasoconstriction can severely decrease the ability of surface cooling to achieve the desired core temperature. This can be partly addressed by administration of anesthetics or alpha-2 adrenergic agonists, but these drugs produce other significant management is-

sues such as hypotension. In the obese, central cooling may be the only practical way of reaching the desired target temperature in a reasonable period of time. Also, body temperature may undershoot the target. Rewarming requires time and may not always be achievable within appropriate time frames; this limits the degree of hypothermia that can be used in shorter surgical procedures. In the International Hypothermia for Aneurysm Surgery Trial, a significant number of patients cooled to 33° C could not be returned to normothermia by the end of surgery and required rewarming in the intensive care unit. This resulted in prolonged tracheal intubation, mechanical ventilation, inability to determine the presence or absence of neurologic deficits, and caused problems with coagulation (personal observations). Better methods of noninvasive cooling and warming of patients are needed.

It is a reasonable proposition that, within limits, greater degrees of hypothermia are associated with greater degrees of protection. However, significant problems confront non-hibernating mammals at body temperatures below 32° C. Myocardial irritability, leading to ventricular fibrillation, is a major problem at 32° C and below. Alaphastat acid base management may increase myocardial stability (Swain et al., 1984). Respiratory depression is another major problem at low temperature in non-cold-tolerant species (Milsom et al., 2001).

Hypothermia in both cold-tolerant and non-cold-tolerant mammals is associated with blood coagulation problems, particularly platelet dysfunction. Platelet dysfunction may persist after rewarming. The mechanisms of cold sensitivity of platelets have been recently determined and may be targeted for modulation (Hoffmeister et al., 2003). Presently, hypothermia can result in excessive blood loss and use of blood products. These factors add to the risks and expense of health care.

Shivering may accompany hypothermia in nonanesthetized or sedated patients. Myocardial ischemia induced by the shivering is poorly tolerated by individuals with coronary artery disease. While shivering can be prevented with drugs such as meperidine or clonidine, additional risk and need for monitoring is created.

What Limits the Hypothermia Tolerance of Mammalian Tissues?

A major factor limiting the clinical application of hypothermia is our limited knowledge of what limits cellular tolerance of hypothermia. It is a reasonable guess that the hypothermia intolerance of mammalian cells contributes to the unacceptably high incidence of severe complications associated with surgeries in-

volving hypothermia. Given that we know that cells from nonhibernating mammals have limited tolerance of hypothermia, can we be sure that hypothermia itself is not part of this problem? For example, if we could improve the hypothermia tolerance of the human brain, could we reduce the incidence of neuropsychiatric deficits following cardiac surgery from 30% to 5%? Could we use more profound hypothermia for longer periods to reduce complications related to treating congenital heart disease? Also, it is reasonable to assume, based on a wide variety of evidence, that the intrinsic hypothermia intolerance of tissues from nonhibernating mammals is a significant issue that limits the preservation of transplant organs. To what extent this intolerance produces problems that counteract some of the benefits of hypothermia for these organs is not clear.

Most mammalian cells withstand hypothermia for short periods of time, as long as ice is not formed. Significant limitations to the tolerance of cells to prolonged hypothermia include membrane lipid dysfunction, energy production/consumption imbalance and ionic dysregulation (Rubinsky, 2003). In cold-stressed cells, $\text{Na}^+\text{-K}^+$ ATPase function decreases below the level needed for regulation of transcellular Na^+ and K^+ gradients. Cells accumulate Na^+ and water and leak K^+ . Cellular edema and electrolyte imbalance is common in profoundly hypothermic humans. Ion channels in general have a low energy of activation and are less temperature-dependent than many other aspects of cellular function—i.e., ionic conductance decreases more slowly than energy production as temperature drops (Hochachka, 1986). This has significant consequences because the ion pumps that are required to restore ionic gradients are quite temperature sensitive due to their reliance on ATP production. In contrast, hypothermia tolerant cells can effectively reduce ionic leaks at low temperature (Boutilier, 2001). In nontolerant cells, movements of monovalent cations may lead to membrane depolarization, opening of ion channels, and Ca^{2+} influx. As in hypoxic or ischemic cells, calcium homeostasis is a major issue affecting short and long-term survival. The lack of information related to the role of Ca^{2+} in triggering cold injury (although calcium may play a role in hypothermic preconditioning), and the activation of apoptotic machinery is notable. Lessons learned from how the cells of hibernators manage Ca^{2+} homeostasis may be extremely important in designing strategies to improve cellular hypothermia tolerance. Some of these mechanisms were recently reviewed (Wang et al., 2002).

Conclusions

Hypothermia shows great promise to improve outcome in a variety of ischemic diseases. Recent successes in clinical trials of cardiac arrest will increase interest in expanding the uses of hypothermia to other diseases. However, the full potential therapeutic of hypothermia will not be realized until the cellular and molecular benefits of hypothermia are appreciated and the limits of hypothermia tolerance in mammalian cells are better defined.

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Hibernation in Mammals: A Model for Alzheimer-type Phosphorylation of the Microtubule-associated Protein Tau

THOMAS ARENDT,¹ JENS STIELER,¹ ARJEN M. STRIJKSTRA,³ ROELOF A. HUT,³
EDDY A. VAN DER ZEE,² MAX HOLZER,¹ AND WOLFGANG HÄRTIG⁴

¹ Paul Flechsig Institute of Brain Research, Department of Neuroanatomy, University of Leipzig, Leipzig, Germany

² Department of Molecular Neurobiology, University of Groningen, The Netherlands

³ Department of Animal Behavior, University of Groningen, The Netherlands

⁴ Paul Flechsig Institute of Brain Research, Department of Neurochemistry, University of Leipzig, Leipzig, Germany

Abstract: The process of neurodegeneration in Alzheimer's disease (AD) and related disorders is associated with the formation of neurofibrillary tangles (NFTs) in vulnerable neurons. NFTs are made up by the microtubule-associated protein tau in a hyperphosphorylated form. The process of tau phosphorylation, therefore, is believed to be of critical importance for NFT formation and its potential link to neurodegeneration. However, the regulation of tau phosphorylation is not understood very well, mostly because of the lack of a physiological *in vivo* model of Alzheimer-type tau phosphorylation. Here we describe during hibernation the formation of highly phosphorylated tau, containing a number of phospho-epitopes typically found in Alzheimer's disease. Alzheimer-type phosphorylation of tau was most intense in cortical pyramidal neurons, which also show the highest vulnerability towards neurofibrillary degeneration in the human brain. Tau phosphorylation in hibernating animals, however, was not associated with fibril formation and was fully reversible after arousal. The repeated formation and removal of Alzheimer-type tau phospho-epitopes might, thus, represent a physiological mechanism not necessarily associated with pathological

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effects. Hibernation will, therefore, be a valuable model to study the regulation of Alzheimer-type tau phosphorylation and its cell biological sequelae under physiological *in vivo* conditions.

Introduction

Neurofibrillar degeneration, i.e., neuronal degeneration associated with the intracellular formation of twisted fibrils made up by the microtubule-associated protein tau, is a neuropathological feature of Alzheimer's disease (AD). These fibrils, commonly referred to as paired helical filaments (PHFs), contain the microtubule-associated protein tau in a hyperphosphorylated form (Goedert et al., 1992). Tau phosphorylation is thus believed to contribute critically to the formation of PHFs. The intensity of PHF formation in the cerebral cortex and hippocampus correlates with the premortem severity of cognitive dysfunction (Arriagada et al., 1992; Holzer et al., 1994). Current concepts of AD, therefore, are based on the assumption that understanding the process of PHF-like phosphorylation of tau is a prerequisite for the development of neuroprotective and therapeutic strategies. Major progress in this area is hampered by the lack of appropriate animal models that would allow us to study the mechanisms involved in the phosphorylation and dephosphorylation of a PHF-like phosphorylation pattern of tau under *in vivo* conditions.

Here we demonstrate that hibernation in European ground squirrels is associated with Alzheimer-like phosphorylation of tau in those brain areas and neuronal types that are vulnerable to neurofibrillary degeneration in the human brain. Tau phosphorylation in hibernating animals is fully reversible very rapidly during euthermy. Hibernation will, thus, represent a model to study the regulation of Alzheimer-like tau phosphorylation and its cell biological sequelae under physiological *in vivo* conditions.

Materials and Methods

Animals

European ground squirrels (*Spermophilus citellus*) were kept in lucent cages (length x width x height = 48 x 28 x 50 cm) with a wooden nestbox attached (15 x 15 x 15 cm) and food (rabbit breeding chow, Teurlings, Waalwijk, The Netherlands) and water supplied *ad libitum*. Hibernation was induced by gradually lowering ambient temperature from 20° C to 7° C and changing light conditions from light:dark = 12:12 h to continuous dim red light (< 1 lux) in autumn. Individual torpor-arousal patterns were assessed by measuring

nestbox temperatures every minute with a computer-based recording system or by recording outside nestbox activity. Registration of torpor-arousal patterns was validated by using customized abdominal temperature loggers (Tidbit, Onset, USA) that registered body temperature every 48 minutes. This study was approved by the Animal Experiments Committee of the University of Groningen (BG02198).

Animals were sacrificed in four different stages within the torpor and arousal periods in hibernation: torpor short (TS, $n = 6$), torpor long (TL, $n = 6$), arousal short (AS, $n = 6$), arousal long (AL, $n = 7$). Eleven animals were sacrificed after becoming continuously euthermic after hibernation (EU, $n = 11$). Hibernating animals showed hypothermic periods of (mean \pm -SEM) 11.01 \pm 0.18 days and euthermic periods (including arousal) of 21.13 \pm 0.38 hours before sacrificing. Brain material of the hibernating animals was collected after 1.53 \pm 0.06 h (AS), 8.27 \pm 0.05 h (AL), 2.34 \pm 0.21 days (TS) and 7.11 \pm 0.1 days (TL) following the onset of the previous (final) arousal to euthermia. Body temperatures upon start of perfusion confirmed that the groups represented the specific phases of hibernation: rectal temperatures were 30.9 \pm 1.6 $^{\circ}$ C (AS), 34.5 \pm 0.3 $^{\circ}$ C (AL), 9.8 \pm 1.5 $^{\circ}$ C (TS), and 8.21 \pm 0.3 $^{\circ}$ C (TL). Arousal was induced by gentle handling at room temperature for three to five minutes. Arousal induction was carried out at least 10 weeks after onset of hibernation. At the time of brain material collection, the duration of the previously experienced torpor phase did not differ between the groups. Brain material of nonhibernating animals (EU) was collected six to seven days after cessation of hibernation, initiated by an increase in ambient temperature from 7 $^{\circ}$ C to 25 $^{\circ}$ C in early spring. Animals were transcardially perfused with 4% paraformaldehyde in phosphate buffer.

AD Cases

For comparison to hibernating ground squirrel brain tissue, human tissue of Alzheimer patients was used. The clinical diagnosis of the AD patients was based on the occurrence of significant intellectual dysfunction, i.e., the presence of deficits in at least four aspects of cognitive and social behaviour. Other causes of dementia were excluded by medical, psychiatric, and paraclinical examination (Diagnostic and Statistical Manual of Mental Disorders, DSM-III-R, American Psychiatric Association). Each case met the National Institute of Neurologic and Communicative Disorders and Stroke (NINCDS), Alzheimer's Disease and Related Disorders Association (ADRDA), and Consortium to Establish

a Registry for Alzheimer's Disease (CERAD) criteria for definite diagnosis of Alzheimer's disease, based on the presence of NFTs and neuritic plaques observed in the hippocampal formation and neocortical areas. The whole procedure of case recruitment, acquisition of patients' personal data, performing the autopsy, and handling the autoptic material has been approved by the responsible Ethical Committee of Leipzig University. Tissue blocks of the temporal cortex (Brodmann area 22) were fixed by immersion in 4% paraformaldehyde in phosphate buffer.

Immunohistochemistry

Tissue was cryoprotected in 30% sucrose and 25 μ m frozen sections were cut. For the phosphorylation dependent detection of tau, sections were probed with the monoclonal antibodies AT8, AT180 and AT270 (1:2000, Innogenetics) and subsequently processed with a streptavidin/biotin technique and nickel-enhanced diaminobenzidine as chromogen.

Western Blotting

Western blotting was performed using protein preparation of human cortical brain material of two control and two AD cases (Braak stadium V–VI) and cortical brain material of the European ground squirrel (six to seven animals per group). The material was homogenized in lysis buffer (20mM Tris/HCl, pH 7.2, 2mM MgCl₂, 100mM NaCl, 5mM NaF, 1mM Na₃VO₄, 0.5% NP-40, 1mM DTT, 100 μ g/ml PMSF, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 600nM okadaic acid). Extracted proteins were separated on 10% sodium dodecylsulfate (SDS) polyacrylamide gels using 30 μ g protein per lane and subsequently blotted to a transfer membrane. Membranes were probed with AT8, AT180 (1:1000; Innogenetics), Tau-1 (1:1000; Roche), and PHF-1 (1:2000; courtesy of P. Davies, New York). Detection of bound primary antibodies was performed with biotinylated sheep anti-mouse antibody (1:2500; Amersham Pharmacia) and Extravidin Peroxidase conjugate (1:5000; Sigma). Blots were developed with Super Signal West Pico ECL-System (Pierce).

Results

Hibernation in ground squirrels was associated with the formation of an Alzheimer-like pattern of tau phosphorylation. Strongest reactivity for the phosphorylation-dependent detection of tau (AT8) was found in the ventral hippocampus, entorhinal cortex, and isocortex, i.e., brain areas that are constantly very early and most severely affected in AD (Fig. 1). Labeling was also

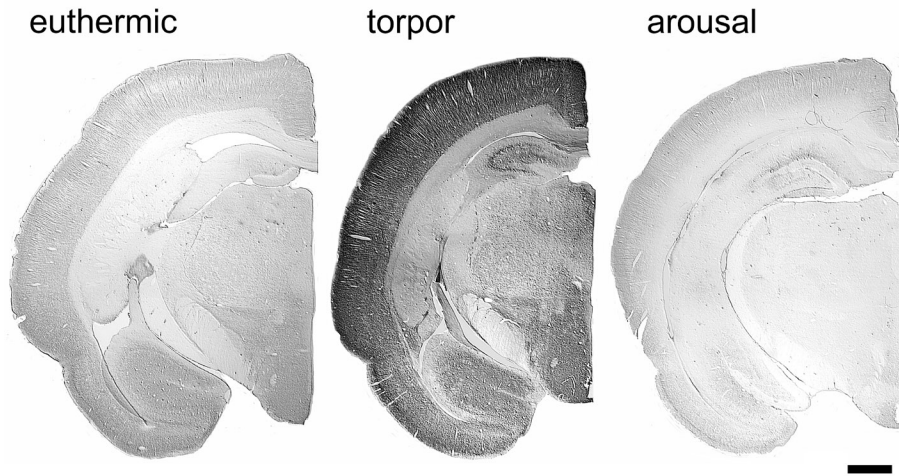


Fig. 1. Immunohistochemical detection of an Alzheimer-type phosphorylation of the microtubule-associated protein tau by the monoclonal antibody AT8 in long torpor (middle) as compared to a nonhibernating euthermic animal (left) and an animal ~7 hours after onset of arousal (right). Immunoreactivity during torpor was most pronounced in the cortical mantle and to a somewhat lesser extent also present in hypothalamic and epithalamic nuclei, is completely reversible after arousal. Scale bar: 1 mm.

seen in subcortical areas, in particular in hypothalamic and epithalamic nuclei, while thalamic nuclei were only marginally reactive.

Hyperphosphorylation of tau during torpor was associated with its subcellular translocation from the axonal to the somatodendritic compartment which corresponds to the subcellular distribution of tau seen in AD. Phosphorylated tau both in cell soma and dendrites of hibernating animals could be labelled by different antibodies that recognize specific PHF-tau epitopes. In AD, these antibodies label neurofibrillary tangles and neuritic components of plaques containing PHFs (Fig. 2).

Effects of hibernation on the Alzheimer-like phosphorylation stage of tau were also analyzed by Western blotting of cortical protein extracts of European ground squirrels (Fig. 3A) compared to human AD cases (Fig. 3B). Consistent enhancement of immunoreactivity is shown for all phosphorylation-dependent antibodies tested, detecting three PHF-like phosphoepitopes on tau (AT8, AT180, PHF-1). Thus, phosphorylation of serines 202, 396, 404, and of threo-

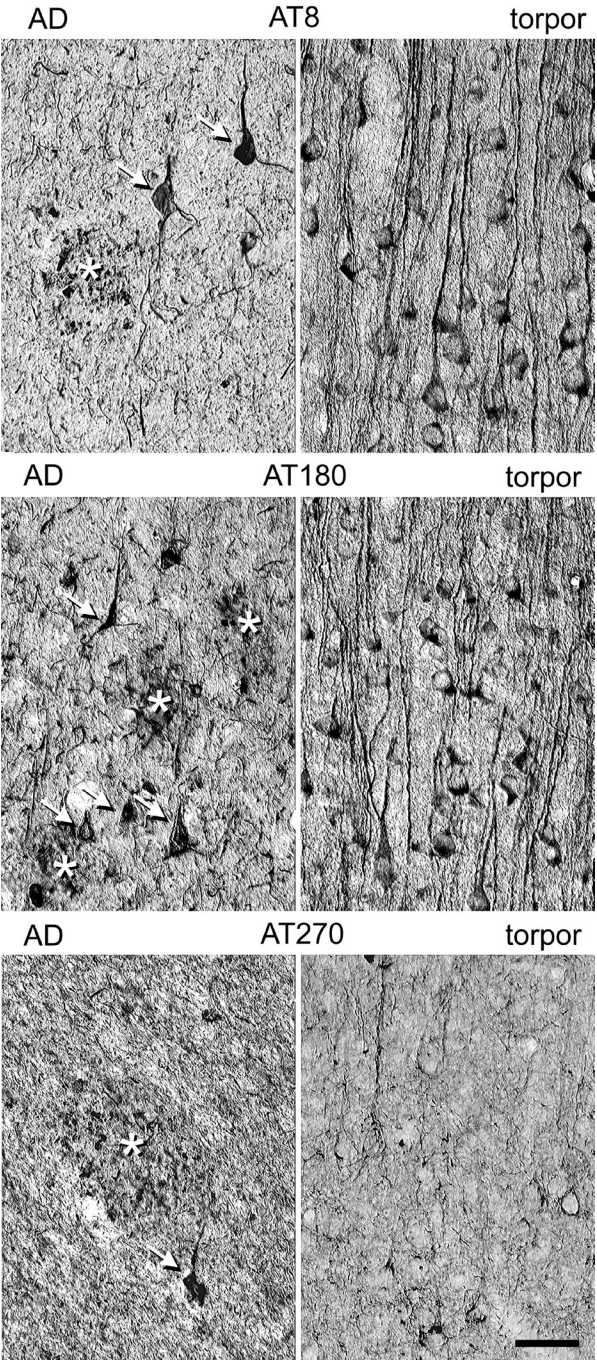


Fig. 2. Phosphorylation-dependent detection of tau in Alzheimer's disease (AD) and hibernation. Cortical pyramidal neurons of torpid animals are reactive for phospho-specific tau antibodies that in AD label neurofibrillary tangles (arrows) and neuritic components of plaques (asterisks) (AT8: Ser202/Thr205; AT180: Thr231; AT270: Thr181). Scale bar: 50 μ m.

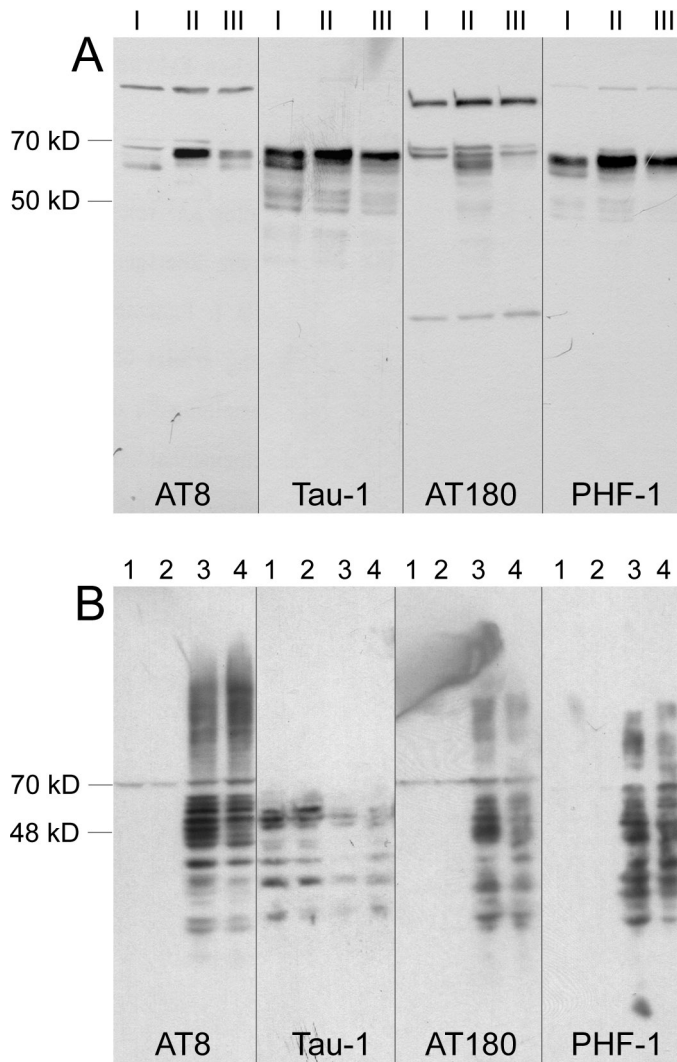


Fig. 3. Western blot analysis of protein extracts of cortical brain material of European ground squirrel (A) and human cortical brain material (B). Blot A: lane I: euthermic animal; lane II: torpid animal after ~7 days of torpor; lane III: aroused animal ~7 hours after onset of arousal. Blot B: lanes 1 and 2 control cases; lanes 3 and 4 Alzheimer cases. Used antibodies are indicated.

nines 205, 231, corresponding to the longest human isoform, becomes highly enhanced in the hibernation cycle during torpor, indicating a large increase in tau phosphorylation. These findings were corroborated by a diminished reactivity of the 68kDa band by Tau-1, which detects tau when dephosphorylated on Ser199/Ser202. In contrast to AD, reactivity for any phosphorylation-dependent antibody used in the study completely disappears after longer periods of arousal.

The formation of hyperphosphorylated tau in AD develops gradually. Phosphorylation-dependent antibodies first label neuropil components. At this stage, cell bodies are still free of tangles and show a diffuse immunolabeling of moderate intensity (Fig. 4A). Subsequently, neurofibrillary tangles are formed by aggregation of hyperphosphorylated tau. Correspondingly, phosphorylation-dependent staining of tau becomes more condensed (Fig. 4B). At most advanced stages, immunoreactive material eventually occupies cell bodies completely (Fig. 4C).

The formation of hyperphosphorylated tau during hibernation also develops gradually. Contrary to AD, however, it is not associated with aggregation of tau and fibril formation. Moreover, tau phosphorylation is completely and very quickly reversible after returning to euthermic stages. At the transition from euthermia to torpor, the formation of PHF-like phosphorylated tau first becomes detectable in the apical dendrite of pyramidal cells, and only very few perikarya are reactive (Fig. 5). Conversely, after arousal, immunoreactivity first disappears in the perikarya while it remains somewhat longer in dendrites (Fig. 5).

Discussion

Hibernation is a physiological strategy of adaptation to inhospitable environmental conditions. To minimize energy expenditure, overall metabolic rate as well as heart and respiratory rate are greatly reduced during hibernation (Geiser and Kenagy, 1988; Wang, 1978), by tolerating a body temperature near the ambient temperature in a regulated hypothermic state. Low metabolic rate is accompanied by dramatically reduced neuronal functions, and electroencephalographic activity is strongly reduced (Shtark, 1970; Daan et al., 1991; Walker et al., 1977; Strijkstra et al., 1999). Correspondingly, hibernation elicits negative effects on memory function (Millesi et al., 2001). Therefore, hibernators may interrupt the torpor state regularly and necessarily return to euthermic arousal episodes, to protect against mechanisms that otherwise would lead to fatal loss of neuronal connectivity (Strijkstra and Daan, 1998; Strijkstra et al., 2003) or functionally important loss of memory.

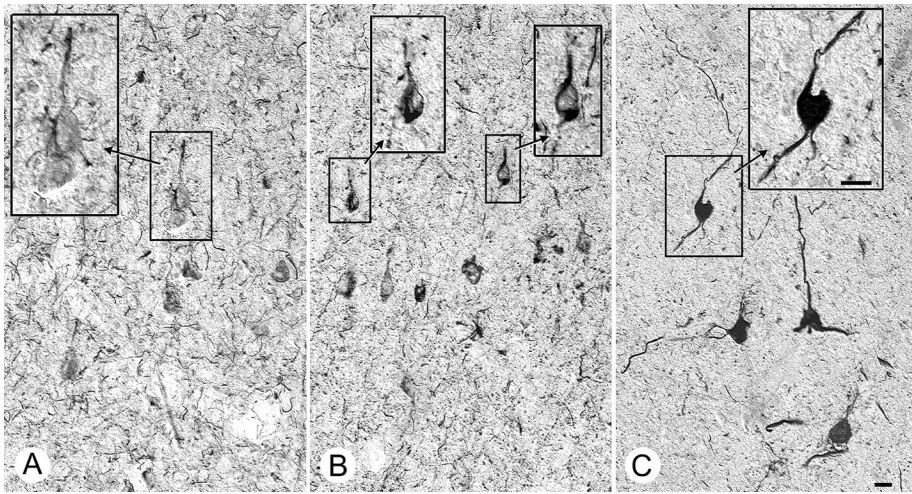


Fig. 4. Developmental sequence of phospho-specific reactivity of tau (AT8) in cortical pyramidal cells during progression of Alzheimer's disease. At initial stages, reactivity is preferentially found in the neuropil, while cell bodies show a diffuse and only rather pale staining (A). With the progressive accumulation of tangle-bearing material in cell bodies, reactivity becomes more intense and condensed (B) and eventually occupies the entire cell body (C). Scale bars: 30 μ m.

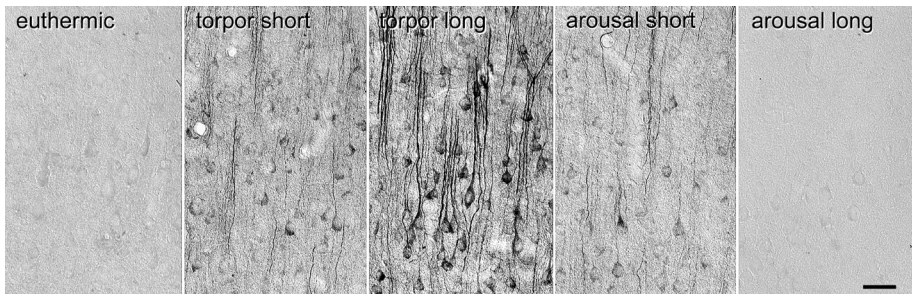


Fig. 5. Developmental sequence of phospho-specific reactivity of tau (AT8) in cortical pyramidal cells during hibernation. Reactivity develops gradually at the transition from euthermia to torpor. It first appears in dendrites, and after longer duration of torpor occupies the entire somatodendritic domain. After arousal, reactivity first disappears in cell bodies, while it remains somewhat longer in dendrites. Reactivity is completely abolished after longer duration of arousal phases.

Suppression of metabolism and thermogenesis during hibernation is critically regulated through reversible phosphorylation of enzymes and proteins that limits rates of flux through metabolic pathways (Storey, 1997). As shown in the present study, this mechanism of reversible protein phosphorylation also affects the microtubule-associated protein tau, thereby generating a condition that in the adult human brain is associated with neurofibrillary degeneration (Arendt et al., 2003). The present results, thus, indicate that regulating tau-(hyper)phosphorylation is preserved in the adult mammalian brain. It apparently reflects a physiological mechanism and is not necessarily associated with pathological effects. On the contrary, increased phosphorylation of tau might even be associated with some neuroprotective action (Arendt et al., 1998; Ihara, 2001). However, the necessity of regular arousal phases to protect against permanent memory loss might indicate the potentially deleterious sequelae of this process if it lasts too long.

Most importantly, PHF-like phosphorylation of tau in the present model is fully reversible through a mechanism that operates naturally in the mammalian brain. This paradigm might, thus, be useful to study the physiological regulation of tau phosphorylation and dephosphorylation critically involved in the process of neurofibrillary degeneration in AD and related conditions.

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Resistance of Livers to Cold Ischemia/Reperfusion Injury During Hibernation: Involvement of Matrix Metalloproteinase and Nitric Oxide Synthase

HANNAH V. CAREY,¹ TIMOTHY M. PIAZZA,¹ SARAH E. DAVIS,¹ SUSANNE L. LINDELL,¹ ANNA DURRANS,² KIERAN CLARKE,² AND JAMES H. SOUTHARD³

¹ University of Wisconsin, Department of Comparative Biosciences, School of Veterinary Medicine, Madison, Wisconsin USA

² University of Oxford, University Laboratory of Physiology, Oxford, England, UK

³ University of Wisconsin, Department of Surgery, School of Medicine, Madison, Wisconsin USA

Abstract. Hibernation in mammals is associated with physiological changes that would be life-threatening for species that maintain continuous euthermia. Identification of mechanisms hibernators use to survive these changes may improve therapeutic approaches to traumatic injury in nonhibernating species. Here we used hibernating ground squirrels as a model for natural resistance to injury induced by extended cold ischemia/warm reperfusion (cold I/R) of organs prior to transplant. Livers harvested from rats, summer squirrels, and torpid hibernators were stored at 4° C for 0–72 hours. Livers were then warm-perfused *ex vivo* for 60 minutes and perfusates collected for measurement of lactate dehydrogenase (LDH) activity (an indicator of liver damage) and matrix metalloproteinase (MMP) activity. Increasing cold storage time had the greatest effect on LDH release in rat livers, followed by summer squirrels and hibernators. MMP-9 activity in perfusates was not affected by storage time in rat or summer squirrels but increased in hibernators after 24–72 hours storage. MMP-9 activity was higher in perfusates from hibernating compared with summer squirrels at 24 and 72 hour storage times. In livers not subject to cold I/R, expression of nitric oxide synthase (NOS) isoforms was higher in hibernating vs. summer squirrels. The results suggest that the hibernating phenotype confers superior resistance to

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cold I/R injury in liver and may represent a global preconditioning response that involves increased expression of NOS.

Introduction

During winter, mammalian hibernators experience a variety of physiological changes that would be life-threatening for species that maintain continuous euthermy throughout the year. These include severe reductions in body temperature (T_b), ventilation, cardiac output, and tissue perfusion that occur as animals cycle into and out of the torpid state. Because blood flow to the splanchnic region is greatly reduced during torpor and rapidly restored during interbout arousals, visceral organs may be at risk for oxidative stress secondary to free radical release when hypoperfused tissues are rapidly reperfused. Studies from our laboratory have provided evidence for oxidative stress in the intestine during hibernation in ground squirrels, although this is not accompanied by obvious signs of tissue damage (Carey et al., 2003c; Carey et al., 2000).

Research in nonhibernating species has shown that resistance to the damaging effects of ischemia/reperfusion can be achieved through the phenomenon known as preconditioning, in which brief episodes of ischemia (or other forms of oxidative stress) render organs resistant to the detrimental effects of a second oxidant challenge. Preconditioning can be acute, with a duration of several hours or delayed, in which protection lasts for up to several weeks. Although most well studied for the heart, several other organs and tissues are now known to undergo preconditioning, including the gut, liver, skeletal muscle, kidney, brain, and vasculature. Evidence from our laboratory and others suggests that hibernation may represent an evolutionarily derived state of natural preconditioning that develops before the start of the hibernation season and is maintained as animals undergo repeated cycles of torpor and arousal (Carey et al., 2003a). If this hypothesis is correct, the challenge is to identify those characteristics of the hibernating phenotype that provide protection against stress and ultimately test their ability to provide protection in stress models using nonhibernating species, including humans.

To identify defense mechanisms that are part of natural preconditioning in hibernators, we used a model of liver cold ischemia/warm reperfusion (cold I/R) that mimics organ preservation prior to transplant. In previous studies we determined whether livers of a hibernating species (ground squirrel) have enhanced protection against cold I/R injury compared with a nonhibernator (rat), and if so, whether cold I/R protection was greater in the hibernating vs. summer

phenotype in ground squirrels (Carey et al., 2003b). In this study, we examined the effects of hibernation and cold I/R on release of matrix metalloproteinases (MMPs) from livers during warm reperfusion. This was of interest because despite their many beneficial roles (Chakraborti et al., 2003; Sternlicht and Werb, 2001), increased release of MMPs has been implicated in the pathogenesis of warm and cold I/R injury in liver and other organs (Upadhyay et al., 1997). In addition, because nitric oxide (NO) is considered to be a key mediator of the preconditioning response (Selzner et al., 2003; Park et al., 2003) and has also been reported to stimulate MMP release (Marcet-Palacios et al., 2003), we determined whether levels of nitric oxide synthase (NOS) isoforms in liver differ in hibernating and active squirrels.

Methods

Livers were harvested from euthermic rats, summer thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), and hibernating squirrels. For cold I/R experiments, hibernators were killed 3–5 days into a torpor bout ($T_b \sim 5^\circ \text{C}$, estimated from rectal temperature probes at time of kill). Livers were used either fresh (0 hours storage) or after storage at 4°C in University of Wisconsin solution for 24 or 72 hours. After the storage period they were mounted in an isolated perfused liver setup and perfused for 60 minutes with warmed (37°C), oxygenated Krebs-Henseleit buffer. Perfusates were collected at 60 minutes and stored at -80°C for later analysis of lactate dehydrogenase (LDH) activity (an indicator of liver damage), bile production (an indicator of liver viability), and matrix metalloproteinase (MMP) activity using gelatin zymography. For the latter, 15 μl of perfusate were electrophoresed in gels that contained 10% polyacrylamide supplemented with 0.1% gelatin and then stained with Coomassie Blue. The MMPs were identified by their ability to digest gelatin (clear bands) and by their molecular masses. The gels were scanned and densitometric evaluation of the clear bands for each perfusate was performed using ImageQuant software. Values were normalized by dividing by the value produced by a control MMP-9 protein that was loaded in a separate lane on each gel, and then expressed as units of MMP/g of liver.

For Western blotting experiments, hibernators that were previously implanted with temperature-sensitive telemeters (Minimitter, Sunriver, OR) were killed during one of four activity states: entrance ($T_b \sim 20\text{--}25^\circ \text{C}$); early torpor (within 48 hours of entering torpor, $T_b \sim 5^\circ \text{C}$); late torpor (at least seven days of continuous torpor, $T_b \sim 5^\circ \text{C}$); and interbout arousal ($T_b \sim 36^\circ \text{C}$, at least three hours

after reaching euthermia). Livers were then harvested and cytosolic proteins separated by SDS-PAGE and transferred to nitrocellulose filters using conventional techniques. Immunoblots containing 100 µg protein per sample were incubated with antibodies raised against endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS) (BD Transduction Laboratories) and signals were quantified using densitometry.

Data within each species/activity group were analyzed by one-way ANOVA and if F values were significant, Fisher's LSD test was used to determine significance of differences between pairs of means. Differences between hibernating and active squirrels were analyzed by t-test. The significance was set at $P \leq 0.05$.

Results and Discussion

The cold I/R model we used here is a well-studied *ex vivo* model for assessment of liver viability in nonhibernating species like the rat. In the present study, release of LDH into liver perfusates (a marker for hepatocellular damage) increased significantly after 72 hours cold I/R in livers from rats and summer squirrels, although the increase in rats (~50-fold) was much greater than in the squirrels (~10-fold) (data not shown; [Carey et al., 2003b]). In contrast, LDH activity of perfusates in torpid hibernators was only modestly increased (2.5-fold) after 72 hours cold I/R, and at this storage time LDH activity was significantly lower (~3-fold) in perfusates from torpid compared with summer squirrels. We also measured bile production as a marker for liver viability after cold storage. Compared with fresh livers, bile production was reduced after 72 hours cold storage in livers from all individuals. However, the effect was greatest in rat and summer squirrel livers (82% and 77% reductions, respectively) and least in torpid squirrel livers (52%). Thus, livers from hibernators showed the most resistance to cold I/R injury as indicated by LDH release and bile production, followed by summer squirrels and rats.

To explore mechanisms responsible for the superior resistance of torpid livers to cold I/R, we measured activity of MMPs in liver perfusates. Matrix metalloproteases (MMPs) comprise a multigene family of enzymes that can be secreted and act extracellularly to remodel the extracellular matrix (ECM), or act to modify cell surface proteins (Chakraborti et al., 2003; Sternlicht and Werb, 2001). These actions make MMPs important regulators of such events as tissue morphogenesis, wound healing, angiogenesis and cell signaling. However, the excessive release of MMPs is associated with a variety of pathological conditions, including atherosclerosis, neoplasia, and injury due to ischemia-reperfusion.

With regard to organ preservation, the inappropriate activation of MMPs has been suggested to mediate some of the destructive events that lead to graft failure after cold I/R in rat livers. For example, gelatinase activity of liver flushes obtained shortly after 24 hours cold storage was significantly higher than in fresh rat livers (Upadhyia et al., 1997). The excessive secretion of MMPs after liver cold I/R is thought to mediate detachment of endothelial cells from the sinusoidal surface via degradation of ECM proteins (Upadhyia and Strasberg, 1999). Thus, we hypothesized that livers of ground squirrels, especially those in the hibernating phenotype, may show decreased release of MMPs upon cold I/R compared with rat livers.

A representative zymogram (Fig. 1) shows the presence of two well-characterized MMPs, designated MMP-2 (gelatinase A, 72kD) and MMP-9 (gelatinase B, 92kD) in perfusates from rat and ground squirrel livers. Preliminary analysis indicated that MMP-2 expression in perfusates was not affected by storage time in any of the groups, nor did its expression vary among species/activity states (Fig. 1; densitometric data not shown).

MMP-9 gelatinolytic activity was not affected by storage time in rat or summer squirrel perfusates (Fig. 2). However, MMP-9 activity in torpid squirrel perfusates increased after 24 and 72 hours storage. MMP-9 activity was significantly greater in perfusates from torpid squirrels compared with summer squirrels at the 24 and 72 hours storage times, with values for 0 hours storage showing a similar (but nonsignificant) trend (Fig. 2). Thus, in our model of cold

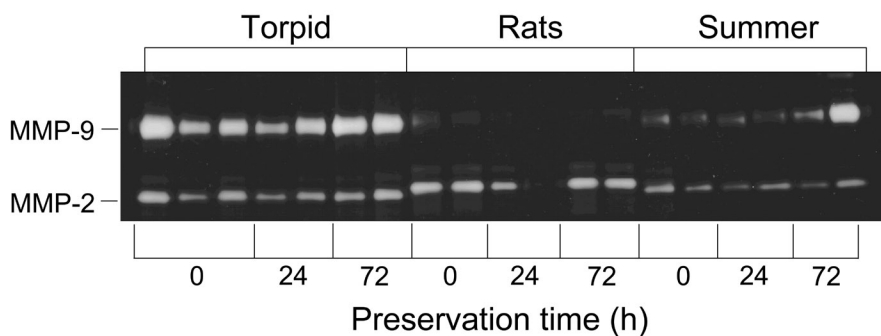


Fig. 1. Representative gelatin zymogram of liver perfusates from ground squirrels and rats. Livers were cold stored for 0, 24, or 72 hours and then reperfused with warm (37° C), oxygenated buffer for 60 minutes. The bands at ~92 and ~72 kDa represent MMP-9 and MMP-2, respectively.

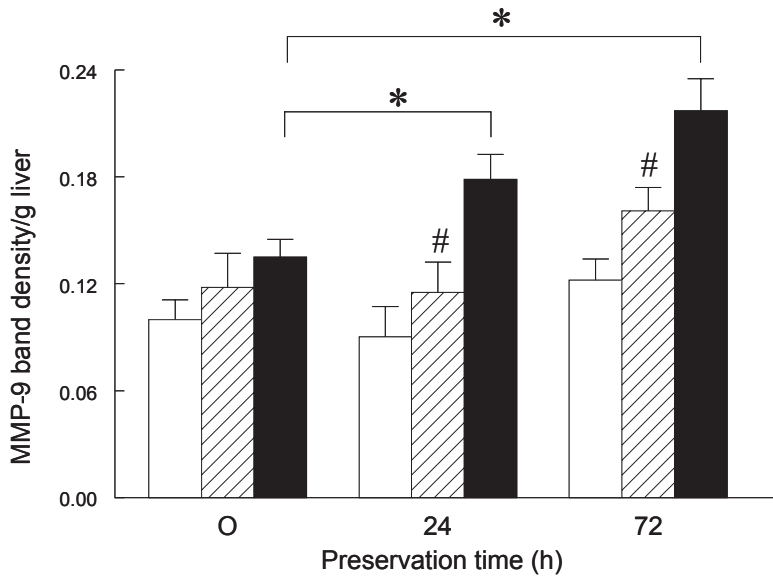


Fig. 2. Densitometric analysis of zymograms for MMP-9 activity in liver perfusates from ground squirrels (summer, hatched bars; hibernators, solid bars) and rats (open bars) subject to cold I/R. Samples sizes (for 0, 24, and 72 hours storage) were rats (5, 7, 6), summer squirrels (7, 6, 7), and torpid squirrels (9, 9, 9). *, For torpid groups, values significantly different ($P < 0.05$). #, Summer values significantly different from torpid ($P < 0.05$).

I/R, increased cold storage time did not result in an increase in MMP release in rat liver perfusates. Although this does not support the conclusion of Upadhyaya et al. (1997), the discrepancy may be due to differences in the protocols used in the two studies. In our study livers were perfused for 60 minutes with 37° C buffer prior to collection of perfusates, whereas Upadhyaya et al. (1997) measured MMP activity in liver flushes collected immediately after the storage period. The MMP-9 activities shown in Fig. 2 were normalized to liver mass, which were similar or slightly lower in the torpid (compared with summer) squirrels used in these experiments. Thus, differences in liver mass cannot account for the higher MMP-9 release in perfusates from torpid squirrels.

The results reported here are interesting for two reasons: (1) only torpid squirrel livers showed increased release of MMP-9 with increasing storage time, and (2) MMP-9 release was greater in torpid than in summer squirrels, particularly for livers that had been cold stored. These effects were unexpected, given

that increased release of MMPs has been linked to cold I/R injury in previous studies, yet cold storage had the least effect on LDH release and bile production in hibernator livers among the groups we studied. If MMP-9 release was involved in liver injury after extended cold I/R, one would expect to see increased proteinase activity in liver perfusates from the two more vulnerable groups (rats and summer squirrels). This raises the possibility that the greater release of MMP-9 in torpid vs. summer squirrel livers, as well as the increase in MMP-9 release with increasing cold storage time in hibernators, may not be deleterious events but rather may reflect beneficial roles for this proteinase during hibernation. Despite its association with tissue damage and certain disease states, the activation of MMPs is required for a number of critical functions, including angiogenesis, wound healing, degradation of inflammatory mediators, and neuronal plasticity (Chakraborti et al., 2003; Sternlicht and Werb, 2001; Kaczmarek et al., 2002). One or more of these effects may be important for hibernation. In fact, recent studies suggest that the therapeutic use of MMP inhibitors in experimental disease models and clinical trials has been disappointing, suggesting that MMP activity in pathophysiological states can be both deleterious and beneficial (Selzner et al., 2003).

The activity of MMPs is regulated by proteins known as tissue inhibitors of metalloproteinases (TIMPs), and the balance of MMP and TIMP activities at local sites is responsible for the functional effect of an MMP on ECM matrix degradation and other proteolytic events (Chakraborti et al., 2003; Sternlicht and Werb, 2001). Thus, it is possible that the greater release of MMPs we observed in liver perfusates from torpid squirrels compared with summer animals may be balanced by increased expression of one or more TIMPs. However, preliminary studies using Western analysis suggest that expression of TIMP-1, a major endogenous inhibitor of MMP-9 is similar in liver tissue of summer and hibernating squirrels (data not shown). The serum activity of MMPs is also influenced by several pan protease inhibitors, including α 2-macroglobulin (α 2M), whose expression is increased in liver and serum during the hibernation season at the mRNA and protein levels (Srere et al., 1992). Upregulation of α 2M during the hibernation season is thought to facilitate blood flow during bouts of torpor via α 2M's effect on inhibiting the clotting cascade. The results presented here suggest that limitation of excessive MMP release after long periods at low T_b may be another role for α 2M in hibernators in addition to its effects on the clotting cascade.

Nitric oxide, particularly when produced by the action of inducible NOS (iNOS), has been shown to stimulate expression of MMP-9 in vascular smooth muscle (Marcet-Palacios et al., 2003). To determine if increased MMP-9 release in hibernator livers is associated with elevated NOS expression, we measured protein levels of iNOS as well as the constitutive NOS isoform typically produced in endothelial cells (eNOS). Western blot analysis indicated there were no significant differences among the four hibernation states in expression of either isoform (data not shown), thus all hibernation values were combined. A nearly five-fold increase in iNOS expression was observed in hibernating squirrel livers, whereas liver eNOS increased ~1.7-fold (Fig. 3). These results therefore provide a possible mechanism for the unexpected release of MMP-9 in livers of torpid squirrels, although further studies using inhibitors of NOS isoforms are needed to confirm that conclusion. Furthermore, because iNOS has been implicated in delayed preconditioning responses in several organs including the liver (Park et al., 2003; Selzner et al., 2003), these findings lend support to the hypothesis that the hibernating phenotype may represent a state of endogenous preconditioning that protects tissues from oxidative or other damage induced by torpor-arousal cycles.

In conclusion, the results of this study suggest that the hibernating phenotype confers superior resistance to cold I/R injury in liver and may represent a global

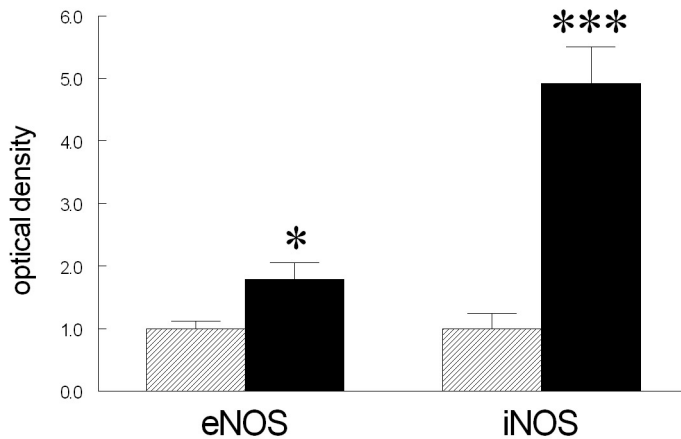


Fig. 3. Densitometric analysis of Western blots for NOS protein expression in liver of summer (hatched bars) and hibernating (solid bars) ground squirrels. $N = 15$ animals per group for iNOS and 12 animals per group for eNOS. *, significantly different from summer, $P < 0.05$; ***, $P < 0.001$.

preconditioning response that involves increased expression of NOS, which has been implicated in delayed preconditioning responses in several organs. Although the functional significance of increased MMP-9 release from liver during hibernation is not yet known, it may play a role in adaptive events such as wound healing and degradation of proteins involved in the inflammatory response.

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Anti-Proliferative Effects of Plasma from Hibernating Rodents

DONNA G. SIECKMANN,¹ DECHENG CAI,² HOWARD JAFFE,³ JOHN HALLENBECK,²
AND RICHARD M. MCCARRON^{1, 4}

¹ Resuscitative Medicine Department, Naval Medical Research Center, Silver Spring, MD 20910-7500, USA

² Stroke Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892-4128, USA

³ LNC-NINDS Protein/Peptide Sequencing Facility, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892-4128, USA

⁴ Department of Pathology, USUHS, Bethesda, MD 20814, USA

Abstract. Hibernation is a natural process with considerable potential for discovery of new therapeutic avenues. Characterization of the regulatory mechanisms involved in hibernation could lead to beneficial strategies for inducing hypometabolism and treating medical problems caused by ischemic conditions. We demonstrate here that hibernating woodchuck or ground squirrel plasma contains a factor(s) that suppresses mitogenic stimulation of mouse spleen cell cultures. This finding suggests a possible mechanism by which hibernating animals maintain a state of immune hypo-responsiveness, thereby protecting cells and tissues from inflammatory-induced ischemic cell death.

Introduction

Ischemia induced by severe hemorrhage or by obstruction of blood flow (stroke or myocardial infarction) triggers a variety of pathophysiological processes which, if not promptly halted, lead to death of cells and tissues. Cell death may occur during the initial ischemic insult when blood flow is considerably diminished, or later as a result of reoxygenation during resuscitation and reflow (reperfusion injury). Hypoxia induces a number of changes (i.e., up-regulation of

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adhesion molecule expression and activation of proteolytic enzymes) that result in activation of inflammatory pathways and generation of reactive oxygen species (ROS), contributing to secondary reperfusion-mediated tissue injury.

A novel strategy for discovery of therapies to deal with the destructive processes set into motion by hypoxia/ischemia is to identify mechanisms or mediators in hibernating animals that confer tolerance to such conditions. In hibernation, the mitotic activity of cells and tissues is dramatically decreased; mitoses are absent or are displayed as atypical forms (Kolaeva et al., 1980). Flow cytometric studies of different tissues of the hibernating ground squirrel (*Citellus suslicus*) have shown that during the entire hibernation bout, there is an accumulation of cells of various organs in the G₁ phase (Kolaeva et al., 1980). A direct examination of DNA synthesis in lymphoid tissue taken from hibernating hamsters demonstrated a substantially reduced synthesis of DNA (Manasek et al., 1965). More recent studies have found antiproliferative activity in extracts of tissues and plasma from hibernating animals. These studies indicate that: (1) brown fat from hibernating ground squirrels (*Citellus undulatus*) blocked proliferation of mouse lymph node cells (Atanassov et al., 1995); (2) aqueous extracts of brain tissue of thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) inhibited ³H-thymidine uptake of Chinese hamster ovary cells (Amorese et al., 1982); (3) acetic acid extracts of the small intestine of hibernating ground squirrels (*Citellus undulatus*) inhibited embryonic development of sea urchin eggs (Kramarova et al., 1992); and (4) albumin containing fractions of hibernating woodchuck plasma inhibited growth of tissue cultured cells (Chien and Oeltgen, 1993). All of these data suggest that cell division (and its inherent link to cellular metabolism) may not only be involved in hibernation, but could serve as a functional assay for screening factors in the plasma that regulate metabolism. Preliminary results (McCarron et al., 2001) demonstrating quantitative changes in numerous plasma proteins indicated the potential utility for a high throughput assay to screen individual plasma samples as well as fractions obtained by chromatographic separation. Recently, a mitogen-stimulated mouse spleen cell proliferation assay run on a 96-well microtiter plate format was devised to screen various plasma samples for factor(s) that inhibit cell proliferation. This assay and initial results using whole and fractionated plasmas from hibernating squirrels and woodchucks is described here.

Materials and Methods

Animals

Thirteen-lined ground squirrels (*Citellus tridecemlineatus*) trapped in late summer were obtained from a certified supplier (TLS Research, Chicago, IL). They were fed rodent chow and held in a conventional animal room or an environmental chamber in the dark at 4° C (hibernaculum). BALB/cByJ mice were purchased from the Jackson Laboratories, Bar Harbor, ME, and housed conventionally in cages supplemented with rodent chow and water *ad lib*. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and were approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee.

Plasma Samples

Plasma samples were collected between January and April from hibernating (HIB) squirrels or nonhibernating squirrels maintained in the conventional animal room all winter (winter-active, WA); plasma samples were also obtained from summer-active (SA) squirrels in early September. Heparinized blood samples were obtained by cardiac puncture from anesthetized WA or SA squirrels or HIB squirrels that had been hibernating for ≥ 2 days. Individual squirrel samples were centrifuged at 600 $\times g$ and stored at -80° C. Experiments tested plasma preparations pooled from eight HIB, eight WA, or four SA squirrels. Plasma samples from individual HIB (December or February) and active (May or September) woodchucks (*Marmota monax*) were purchased from Northeastern Wildlife, Plymouth, NY.

Cell Culture Assays

In the proliferation assay, 5×10^5 BALB/cByJ mouse spleen cells in 100 μ l Minimal Essential Medium Eagle (Biowhittaker, Walkersville, MD) containing 0.01 M Hepes buffer, 1 mM sodium pyruvate, 2 mM glutamine, 1 mM nonessential amino acids, 0.01 μ M 2-mercaptoethanol, 0.75% sodium bicarbonate, 5 μ g/ml gentamicin, and 10% fetal calf serum (HyClone, Login, UT) were cultured in triplicate in 96-well microtiter plates (Costar, Corning Life Sciences, Acton, MA) with 1–2 μ g/ml of concanavalin A (Con A, Amersham Pharmacia Biotech, Piscataway, NJ), and 50 μ l of plasma samples or buffer for 48 h at 37° C, in humidified 7.5% CO₂/ 92.5% air. Cultures were pulsed with 1 μ Ci

^3H -thymidine (^3H -TdR, Perkin Elmer Life Sciences, Boston, MA) for the last four hours of culture and collected on filter mats using a cell harvester (MACH III M, TomTech, Orange, CT). Radioactive uptake was measured in a Wallac Microbeta Tri-Lux scintillation counter (Perkin Elmer Life Sciences).

Separation of Plasma Samples by HPLC

Plasma samples were clarified by centrifuging for 15 minutes at 27,000 $\times g$. Individual squirrel plasma samples (100 μl) were diluted with 100 μl of HPLC buffer (0.2 M sodium phosphate buffer, pH 7.0) and fractionated by size-exclusion high performance liquid chromatography (SEC-HPLC) on a Beckman System Gold HPLC, using two Ultraspherogel SEC 2000 columns (7.5 \times 300 mm, Beckman Instruments, Fullerton, CA) and a guard column, eluted at 0.8 ml/min/fraction with HPLC buffer. Fractions were dialyzed with 10 mM Na^+/K^+ PBS, pH 7.4 (PBS, Sigma, St. Louis, MO) and stored at -80°C .

Results

Pooled plasma from HIB or SA animals was added to mouse spleen cultures at a final concentration of 6%, and proliferative responses were assessed. Samples of HIB plasma from either woodchucks or ground squirrels inhibited Con A-induced mitogenesis significantly more than samples from SA animals (Table 1). Results show that Con A induced proliferation was inhibited 88% by HIB woodchuck plasma and 52% by HIB ground squirrel plasma as compared with SA plasma. Addition of the plasma after 24 hours of culture had no effect on the proliferative response (data not shown). This indicated that the inhibition occurred early in the proliferative response and was not due to cell death or passive inhibition of ^3H -TdR uptake.

Table 1. Inhibition of Con A induced proliferation by plasma from hibernating animals. Values represent the mean of triplicate cultures minus the background \pm SE. Control Con A response was $44,529 \pm 3,803$ cpm. Background proliferation was $2,337 \pm 362$ cpm/culture.

	Proliferative Response (cpm/culture)	
	HIB	SA
Woodchuck	$3,838 \pm 518^*$	$33,313 \pm 1,855$
13-lined ground squirrel	$11,952 \pm 1,070^*$	$24,953 \pm 1,101$

*Significantly different from SA at $p < 0.001$.

Separation of HIB squirrel plasma proteins by size exclusion HPLC resulted in the isolation of inhibitory (antiproliferative) activity into one major peak that eluted near the void volume. Fifty μ l aliquots of HPLC SEC fractions from individual plasma samples were added to cultures of Con A-stimulated mouse spleen cells. Only one fraction from separation of HIB plasma samples was inhibitory; the proliferative response was inhibited 98%. None of the WA plasma fractions were inhibitory. The separation range of the size exclusion column and the appearance of the inhibitory protein in a fraction near the void volume suggested that the inhibitory factor was a large molecular weight protein (> 200 kDa).

Discussion

The data demonstrate the presence of a protein factor in the plasma of hibernating thirteen-lined ground squirrels and woodchucks, which suppresses Con A-stimulated proliferative responses. Although total protein levels in HIB and SA plasma samples were similar (assessed by HPLC analysis), the suppression may be due to elevated levels of a specific inhibitory protein in HIB plasma. The identity of this inhibitory plasma protein and the mechanisms by which it might regulate cellular functions during hibernation are currently under investigation.

The study supports the notion that inhibition of lymphocyte activation during hibernation might be a mechanism to suppress inflammatory responses that would otherwise be triggered by the low blood flow conditions or other stress conditions associated with hibernation. Immunosuppression during hibernation is consistent with the requirement to lower energy expenditure and has been documented by other investigators. In hibernating frogs, depletion of lymphocytes and hematopoietic cells from the blood, marrow, and lymphatic organs leads to a decreased antibody-forming cell response (Cooper et al., 1992). Also, hibernating animals are unable to respond to vaccination shortly before (Jaroslow and Smith, 1967) or during (Andjus and Matic, 1959) a bout of hibernation; however, soon after arousal the animals recover their immune competence. Hibernating hamster spleen fragments immunized to sheep red blood cells *in vitro* have a lower antibody response than fragments from nonhibernating hamsters (Sidkey and Auerbach, 1968).

Various reports in the literature suggest that suppression of cellular proliferation may be due to a variety of mediators found in brown fat (Atanassov et al., 1995), intestine (Amorese et al., 1992), brain (Kramarova et al., 1992) or plasma (Chien and Oeltgen, 1993). The plasma factor described here does not appear to be related to any of these previously identified antiproliferative factors

because of its high molecular weight. Although the precise mechanisms responsible for induction and maintenance of hibernation remain to be defined, regulation of lymphocyte activation represents an important element in the maintenance of immune system quiescence. Further studies are planned to determine if this anti-inflammatory factor might protect against cell and tissue damage in an *in vivo* model of ischemia.

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Antifreeze Proteins in Terrestrial Arthropods

JOHN G. DUMAN,¹ VALERIE A. BENNETT,^{1,3} N. LI,^{1,4} L. WANG,¹ L. HUANG,¹
T. SFORMO,² AND B. M. BARNES²

¹ University of Notre Dame, Department of Biological Sciences, Notre Dame, IN, USA

² University of Alaska Fairbanks, Institute of Arctic Biology, Fairbanks, AK, USA

³ Colorado State University - Pueblo, Department of Biology, Pueblo, CO, USA

⁴ Bristol-Myers Squibb Co., Biotechnology Analytical Biochemistry, Cicero, NY, USA

Abstract. Antifreeze proteins (AFPs) are common adaptations of terrestrial arthropods from cold regions. A recent survey of insects and spiders from the interior of Alaska showed that ~26% of the species tested had AFPs. Most AFP-producing species are freeze avoiding and the AFPs function to (1) inhibit inoculative freezing across the cuticle, and (2) inhibit ice nucleators in the hemolymph and gut, thereby promoting supercooling. Overwintering larvae of the beetle *Dendroides canadensis* produce a family of 13 AFPs, each consisting of varying numbers of 12- and 13-mer repeats. Every sixth residue in these AFPs is cysteine, and these are disulfide bridged to produce a very rigid protein that presents threonine-cysteine-threonine repeats in a very regular fashion on one side of the protein such that the threonine hydroxyls are positioned to hydrogen bond to ice. The insect AFPs are by far the most active AFPs known, but optimal activity requires the presence of enhancers, both other proteins with which they interact and low molecular mass solutes such as glycerol. Also, certain *D. canadensis* AFPs (DAFPs) have a synergistic effect on other DAFPs.

Introduction

Antifreeze proteins (AFPs) lower the nonequilibrium freezing point of water while not significantly affecting the melting point, thereby producing a difference between the freezing and melting points that is termed thermal hysteresis

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(DeVries, 1986). According to the generally accepted adsorption-inhibition mechanism of action, AFPs depress the freezing point by adsorbing onto the surface of ice crystals at preferred growth sites (Raymond and DeVries, 1977; Raymond et al., 1989; Knight et al., 1991). This forces crystal growth to occur in highly curved (high free energy) fronts rather than the preferred low curvature (low surface free energy) fronts. Consequently the temperature must be lowered before crystal growth can proceed. Some AFPs appear to adsorb to the ice surface by hydrogen bonding via regularly spaced hydrophilic residues that match the ice lattice (DeVries and Cheng, 1992; Sicheri and Yang, 1995). Hydrophobic and van der Waals interactions may also be involved in binding of some AFPs to ice (Zongchao and Davies, 2002).

AFPs were first discovered in fish where they function to lower the freezing point of the hypo-osmoregulating fish below that of seawater (DeVries, 1971; DeVries and Cheng, 1992; Fletcher et al., 2001). Later studies identified AFPs in many insects (Duman, 1977a, 2001; Zachariassen and Husby, 1982; Duman et al., 1992) and other terrestrial arthropods including centipedes (Tursman et al., 1994), mites (Block and Duman, 1989; Sjursen and Sömme, 2000), and spiders (Duman, 1979a; Husby and Zachariassen, 1980; Duman et al., 2004). AFPs are also common in plants (Urrutia et al., 1992; Griffith et al., 1992a, 1992b; Duman and Olsen, 1993), fungi and bacteria (Duman and Olsen, 1993; Sun et al., 1995). At this time 51 species of insects, six spiders, two mites, and one centipede have been reported to produce antifreeze proteins, generally identified by the presence of the unique thermal hysteresis activity in the hemolymph (Duman et al., 2004). In a recent screen of terrestrial arthropods from the interior of Alaska, 25.9% of 75 species of insects and spiders tested exhibited hemolymph thermal hysteresis (Duman et al., 2004), suggesting that AFPs are a common adaptation to the extreme cold found in this region.

Structures of Insect AFPs

The sequences of AFPs from just three species of insects have been published. Two of these are beetles (*Dendroides canadensis* and *Tenebrio molitor*), and their AFPs are very similar. The third is a Lepidoptera, the spruce budworm *Choristoneura fumiferana* (Tyshendo et al., 1997; Gauthier et al., 1998; Graether et al., 2000). All three AFPs have significantly greater thermal hysteresis activity (THA) than do those of fishes, and none are structurally similar to any known fish or plant AFPs. The sequences of nine *T. molitor* (Graham et al., 1997; Liou et al., 1999) and 13 *D. canadensis* AFPs (DAFPs) (Duman et al., 1998; Andorfer

and Duman, 2000) are now known. These are very similar to one another, both within and between species (Duman, 2001). They consist of varying numbers of 12- or 13-mer repeats with molecular masses of ~7.3–16.2 kDa. Throughout the lengths of the AFPs at least every sixth residue is a cysteine. Fig. 1 shows the sequence of one of the mature *Dendroides* AFPs (DAFP-14) arranged to illustrate the repeats.

Fig. 2 shows the 13 known *D. canadensis* AFPs. Note how the basic repeat structure is maintained in the various DAFP_s, but that some have greater numbers of repeats than others. Also the C-termini of certain DAFP_s (3, 9, 10, 11, 14) is extended beyond the C-terminal proline seen in other DAFP_s (i.e., 1, 2, 4 etc). This and other variations in sequence allow the DAFP_s to be divided into three groups (Group I = 1, 2, 4, 6; Group II = 8, 9, 10, 11, 14; Group III = 3, 5, 7, 12). Disulfide mapping of the DAFP_s showed that all cysteines are disulfide bridged (Fig. 3) (Li et al., 1998b). The disulfide bridges act to stabilize the proteins and properly align the residues which hydrogen bond to ice. Hydroxyl groups of the highly conserved threonine residues are especially likely candidates for hydrogen bonding.

The crystal structure of *T. molitor* AFP was reported by Liou et al. (1999), and showed that the repetitive sequence forms a very regular β helix and the conserved Thr-X-Thr motifs form a flat β sheet, which is probably the ice binding surface. Using the *Tenebrio molitor* AFP sequence as a template, we modeled various DAFP_s. The structure of DAFP-6 is shown in Fig. 4.

			1	2	3	4	5	6	7	8	9	10	11	12	13					
A	Q	Q	C	T	G	G	S	D	C	S	S	C	T	V	A					
B			C	T	N	C	R	N	C	P	N	A	L	T	A					
C			C	T	D	S	T	N	C	Y	R	A	R	T	-					
D			C	T	R	S	T	K	C	N	G	A	L	T	-					
E			C	T	D	S	Y	D	C	F	D	A	D	T	-					
F			C	T	R	S	S	N	C	Y	T	A	K	T	-					
G			C	T	D	S	T	N	C	Y	K	A	M	T	-					
H			C	T	R	S	T	M	C	N	G	A	A	T	-					
I			C	T	D	S	Y	D	C	F	D	A	D	T	-					
J			C	T	R	S	T	N	C	Y	T	A	K	T	-					
K			C	T	G	S	T	N	C	Y	Q	A	T	S	-					
L			C	T	D	S	T	G	C	P	S	S	A	S	V	K	Q	I	K	

Fig. 1. Sequence of the largest *Dendroides canadensis* AFP (DAFP-14) arranged to identify the 12 repeats (A–L) depicting the 12 or 13 positions in the repeats. Note how certain positions are highly conserved.

		POSITION																													
		1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7	8	9	10	11	12	13				
A	1		Q	C	T	G	G	S	D	C	R	S	C	T	V	S		C	T	D	S	Y	D	C	H	N	A	E	T	-	
	2	P	Q	C	T	G	G	S	D	C	R	S	C	T	V	S		C	T	D	S	Y	D	C	H	N	A	E	T	-	
	4	P	Q	C	T	G	G	S	D	C	R	S	C	T	V	S		C	T	D	S	H	D	C	H	N	A	E	T	-	
	6		Q	C	T	G	G	S	D	C	R	S	C	T	V	S		C	T	D	S	H	D	C	H	N	A	E	T	-	
	8		Q	C	T	G	G	S	D	C	R	S	C	T	V	A		C	T	D	S	Y	D	C	Y	N	A	A	T	-	
	9	Q	Q	C	T	G	G	S	D	C	R	S	C	T	V	A		C	T	D	S	Y	D	C	F	N	A	A	T	-	
	10	Q	E	C	T	G	G	S	D	C	R	S	C	T	V	A		C	T	D	S	Y	D	C	F	N	A	E	T	-	
	11		E	C	T	G	G	S	D	C	R	S	C	T	V	A		C	T	D	S	H	D	C	H	N	A	Q	T	-	
	14	Q	E	C	T	G	G	S	D	C	R	S	C	T	V	A		C	T	D	S	Y	D	C	F	N	A	D	T	-	
	3		Q	C	T	G	A	P	D	C	S	A	C	T	T	A		C	T	K	S	Y	D	C	Y	K	A	V	T	-	
	5		V	C	T	G	A	P	N	C	N	A	C	T	T	A		C	T	R	S	T	K	C	N	K	A	V	T	-	
	7		V	C	T	G	A	P	N	C	N	A	C	T	T	A		C	T	R	S	T	K	C	N	K	A	V	T	-	
12		V	C	T	G	A	P	N	C	N	A	C	T	T	A		C	T	R	S	T	K	C	N	K	A	V	T	-		
B	1		C	T	D	C	Q	N	C	P	N	A	R	T	A		C	T	R	S	T	N	C	Y	K	A	K	T	-		
	2		C	T	D	C	Q	N	C	P	N	A	R	T	A		C	T	R	S	T	N	C	Y	K	A	K	T	-		
	4		C	T	D	C	Q	N	C	P	N	A	R	T	A		C	T	R	S	T	N	C	Y	K	A	K	T	-		
	6		C	T	D	C	Q	N	C	P	N	A	R	T	A		C	T	R	S	T	N	C	Y	K	A	K	T	-		
	8		C	T	T	N	C	R	N	C	P	N	A	M	T	A		C	T	R	S	T	N	C	Y	K	A	K	T	-	
	9		C	T	T	D	C	Q	N	C	P	N	A	L	T	A		C	T	R	S	T	N	C	Y	T	A	K	T	-	
	10		C	T	T	N	C	Q	N	C	P	N	A	L	T	A		C	T	R	S	T	N	C	Y	T	A	K	T	-	
	11		C	T	T	N	C	Q	N	C	P	N	A	R	S	T	A		C	T	R	S	Y	D	C	Y	N	A	T	T	-
	14		C	T	T	N	C	R	N	C	P	N	A	L	T	A		C	T	R	S	T	N	C	Y	T	A	K	T	-	
	3		C	T	T	D	C	V	N	C	P	N	A	L	T	A		C	T	D	S	T	N	C	Y	E	K	A	T	A	-
	5		C	T	T	N	C	I	N	C	P	N	A	L	L	A		C	T	N	S	S	D	C	F	Y	K	A	V	T	-
	7		C	T	T	N	C	I	N	C	P	N	A	L	L	A		C	T	N	S	S	D	C	F	Y	K	A	V	T	-
12		C	T	T	N	C	I	N	C	P	N	A	L	L	A		C	T	N	S	S	D	C	F	Y	K	A	V	T	-	
C	1		-	-	-	-	-	-	-	-	-	-	-	-		C	T	G	S	T	N	C	Y	E	A	T	-	A			
	2		-	-	-	-	-	-	-	-	-	-	-	-		C	T	G	S	T	N	C	Y	E	A	T	-	A			
	4		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	A			
	6		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	A		
	8		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	
	9		-	-	-	-	-	-	-	-	-	-	-	-		C	T	G	S	T	N	C	Y	E	A	T	-	A			
	10		-	-	-	-	-	-	-	-	-	-	-	-		C	T	G	S	T	N	C	Y	E	A	T	-	A			
	11		C	T	G	S	T	V	C	H	K	A	Q	T	-	C	T	G	S	T	N	C	Y	E	R	A	T	-	A		
	14		-	-	-	-	-	-	-	-	-	-	-	-		C	T	D	S	T	N	C	Y	K	A	M	T	-	-		
	3		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	5		-	-	-	-	-	-	-	-	-	-	-	-		C	T	G	S	T	N	C	Y	K	A	K	S	-	-		
	7		-	-	-	-	-	-	-	-	-	-	-	-		C	T	G	S	T	N	C	Y	K	A	K	S	-	-		
12		-	-	-	-	-	-	-	-	-	-	-	-		C	T	G	S	T	N	C	Y	K	A	K	S	N	-			
D	1		-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	2		-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	4		-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	6		-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	8		C	T	G	S	T	G	C	Y	Q	A	M	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	9		C	T	D	S	T	N	C	Y	R	A	M	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	10		C	T	D	S	T	N	C	Y	K	A	M	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	11		C	T	G	S	T	G	C	Y	N	A	M	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	14		C	T	D	S	T	N	C	Y	R	A	M	T	-	-	C	T	R	S	T	M	C	N	G	A	A	T	-		
	3		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-			
	5		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-			
	7		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-			
12		-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-				
E	1		C	T	R	S	S	N	C	I	N	A	L	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	2		C	T	R	S	S	N	C	I	N	A	L	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	4		C	T	G	S	S	N	C	I	N	A	L	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	6		C	T	G	S	T	N	C	I	N	A	L	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	8		C	T	R	S	S	E	C	N	G	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	9		C	T	R	S	T	M	C	N	G	A	L	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	10		C	T	R	S	T	M	C	N	G	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	11		C	T	R	S	S	E	C	N	G	A	Q	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	14		C	T	R	S	T	K	C	N	G	A	L	T	-	-	C	T	D	S	Y	D	C	F	D	A	D	T	-		
	3		C	T	R	S	T	N	C	Y	K	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	5		C	T	D	S	T	N	C	L	K	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	7		C	T	D	S	T	K	C	M	L	K	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-			
12		C	T	D	S	T	N	C	L	K	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-				

Fig. 2. Sequences of the 13 known *D. canadensis* AFPs (DAFPs) showing the various repeats (A–L). Residues that are different from DAFP-1 are outlined. More similar DAFP's are grouped, I–III.

K		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		14	C	T	R	S	T	N	C	Y	T	A	K	T	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		11	C	T	G	S	T	N	C	H	R	A	T	-	A	-	-
		14	C	T	G	S	T	N	C	Y	Q	A	T	-	S	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		5	C	A	A	S	T	N	S	F	E	A	T	T	S	-	-
		7	C	T	G	S	T	N	C	F	E	A	T	T	S	-	-
		12	C	A	G	S	T	N	C	F	E	A	T	T	S	-	-
M		1	C	T	D	S	T	G	C	P	-	-	-	-	-	-	-
		2	C	T	D	S	T	G	C	P	-	-	-	-	-	-	-
		4	C	T	D	S	T	G	C	P	-	-	-	-	-	-	-
		6	C	T	D	S	T	G	C	P	-	-	-	-	-	-	-
		8	C	T	D	S	T	G	C	P	-	-	-	-	-	-	-
		9	C	T	D	S	T	G	C	P	S	S	A	S	V	K	Q I K
		10	C	T	D	S	T	G	C	P	S	S	S	V	S	V	K H I K
		11	C	T	G	S	T	G	C	P	S	S	G	A	-	-	-
		14	C	T	D	S	T	G	C	P	S	S	A	S	V	K	Q I K
		3	C	T	N	S	T	G	C	P	P	S	A	Y	I	H	Q I N
		5	C	V	N	S	T	G	C	P	P	P	-	-	-	-	-
		7	C	V	N	S	T	G	C	P	P	-	-	-	-	-	-
		12	C	V	N	S	T	G	C	P	P	-	-	-	-	-	-

Fig. 2. (continued)

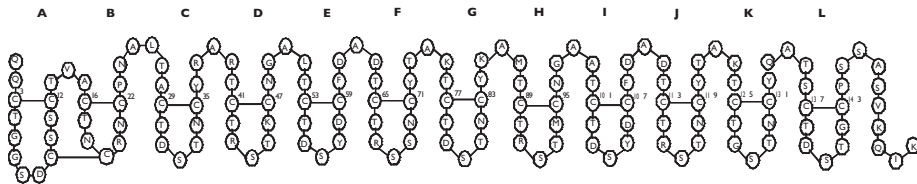


Fig. 3. Sequence of DAFP-14 (Fig. 1) showing the disulfide bridges. Bold letters at the top identify repeats.

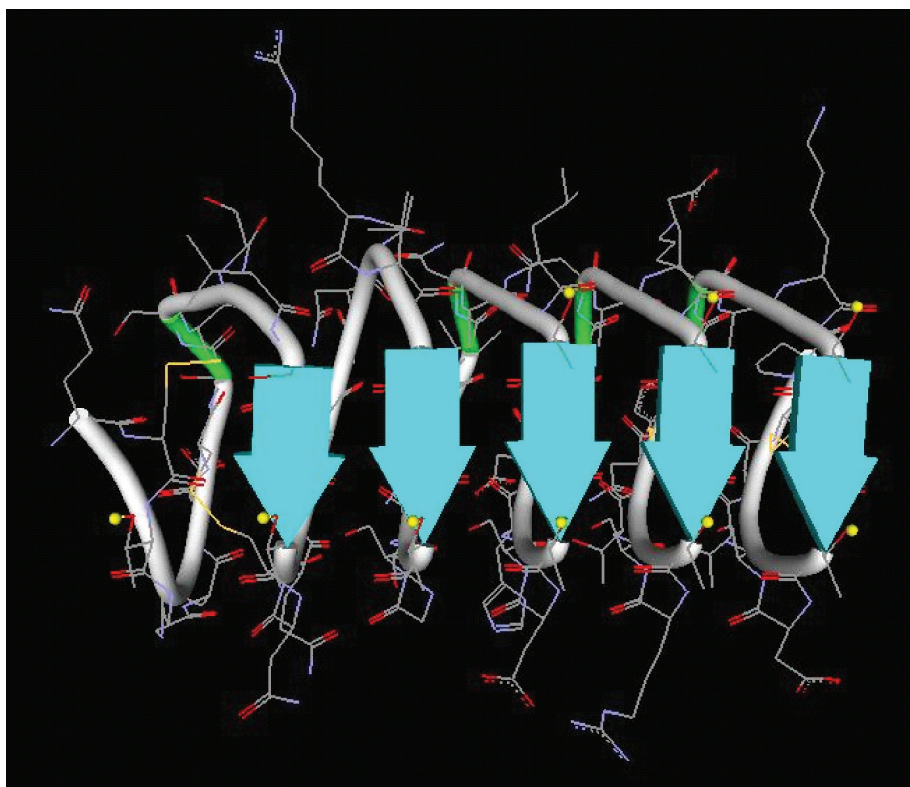


Fig. 4. Molecular model of DAFP-6. Side view with ice binding surface facing the viewer, with the N-terminus at the left. The T-C-T regions of the β sheet (blue arrows) match the ice oxygen spacing (outlined by thin green lines), both in the prism plane (4.48 Angstroms, horizontal spacing in this view) Note the alignment of the threonine side chains (red and yellow) at the tips of the β strands (blue arrows). Disulfide bridges are yellow.

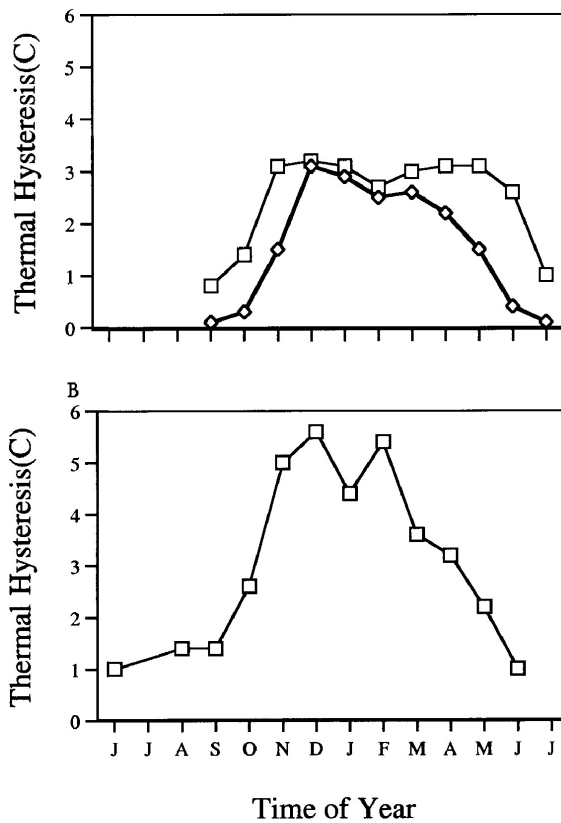


Fig. 5. Variations in hemolymph (□) and midgut fluid (◇) thermal hysteresis activity over the course of two years. (A) The relatively mild winter of 1982–1983. (B) The coldest winter on record in South Bend, Indiana, 1977–1978. (Data from Duman, 1980, 1984).

Control of AFP Activity, Enhancers

AFPs are present in the larvae of the beetle *Dendroides canadensis* in both hemolymph and gut fluid (Duman, 1979b, 1980, 1984), as well as in/on epidermal cells (Olsen et al., 1998). The level of thermal hysteresis activity (THA) indicative of DAFP concentration varies seasonally (Fig. 5). Beginning with low summer values, thermal hysteresis increases slowly throughout the autumn, peaks in midwinter, and slowly decreases through the spring. Also, note that the magnitude of the peak activity varies from year to year, apparently dependant upon the severity of the winter. The winter of 1977–1978 was the coldest on record in this area (northern Indiana and southern Michigan). In contrast, the winter of 1982–1983 was much warmer. Similar THA variations were seen in recent years.

Acclimation of warm adapted insects to low temperature, short photoperiod, short thermoperiod, or in some cases low relative humidity can induce increases in hemolymph THA (Patterson and Duman, 1978; Duman, 1977c; Horwath and Duman, 1984b, 1986). The photoperiodic response is dependant on the entrainment of the circadian system by the light cycle (Horwath and Duman, 1982, 1984). Juvenile hormone stimulates AFP production (Horwath and Duman, 1983; Xu and Duman, 1991; Xu et al., 1992).

THA varies directly with DAFP concentration, and therefore initially it was logical to assume that seasonal or annual (Fig. 5) variations in activity were due entirely to changes in DAFP concentrations. However, DAFP-1 at a concentration of 50 mg/ml produces only $\sim 2.8^{\circ}$ C of THA. Therefore, the high levels of THA seen in even a moderate winter require extremely high concentrations of DAFPs. While DAFP production, as measured by mRNA levels, does correlate with seasonal variations in thermal hysteresis (Andorfer and Duman, 2000), peak hemolymph concentrations of DAFPs were only 1.6–2.6 mg/ml over the last three winters. While more severe winters may trigger higher DAFP concentrations, it is unlikely that all of the variations in activity seen in Fig. 5 are due to changes in DAFP concentrations.

To achieve the THA levels seen in winter hemolymph the presence of AFP enhancers is required. The first enhancers identified were certain proteins to which AFPs were able to bind (Wu and Duman, 1991; Duman et al., 1992). Interestingly, these enhancers had ice nucleating activity, suggesting that the AFPs bind to the ice nucleators, thereby inhibiting them, and in the process the THA of the AFPs is enhanced. The mechanism of enhancement probably results from the ability of the AFP-protein enhancer complex to block a larger surface area of the seed crystal than the AFP alone. Enhancement of THA activity by specific antibodies to *D. canadensis* AFPs lends proof for this mechanism of enhancement (Wu et al., 1991). Also, several low molecular mass THA enhancers were identified (Li et al., 1998a). The best of these is citrate, which increased the THA of an aqueous solution of purified *D. canadensis* AFP from 1.2° C in its absence to 6.8° C. The concentration of low molecular mass enhancer necessary for significant enhancement is typically 0.25–0.50 M. Obviously, physiological concentrations of citrate are much lower. However, normal winter hemolymph concentrations of another enhancer, glycerol, are sufficient to permit it to function as an endogenous enhancer. Studies conducted over recent warm winters showed that both glycerol and protein enhancers contribute significantly to the THA in the hemolymph of *D. canadensis*, raising hemolymph THA by 35–55% and 60–67%

respectively (Duman and Serianni, 2001). In addition, these enhancers greatly increase the abilities of DAFPs to inhibit ice nucleators (Duman, 2002).

To identify proteins and peptides to which DAFPs bind (primarily enhancers and/or ice nucleators), the yeast two-hybrid system was employed using specific DAFPs as “bait” to screen a *D. canadensis* library to identify *in vivo* DAFP-peptide interactions. Several candidate proteins have been identified, and when certain of these were expressed (in an *E. coli* expression system) and added to DAFPs, the THA was increased significantly. In addition, the yeast two-hybrid system identified certain other DAFPs as interacting with DAFP “bait,” indicating that various DAFPs may enhance the THA of certain other DAFPs. Also, by holding the total DAFP concentrations constant and varying the types of DAFPs present, it was demonstrated that certain DAFPs do indeed synergistically enhance the THA of one another.

Functions of AFPs

Freeze Avoidance

DAFPs in freeze avoiding *Dendroides canadensis* larvae function to prevent lethal ice formation by (1) inhibiting ice nucleators, both in the hemolymph and gut, thereby promoting supercooling (Olsen and Duman, 1997a, b; Duman, 2002), and (2) by inhibiting inoculative freezing from external ice across the cuticle (Olsen et al., 1998). It is important to note that the level of protection afforded by DAFPs greatly exceeds the magnitude of THA measured in the insect's hemolymph, typically 3–6° C in midwinter. For example, DAFPs at a concentration which produced just 2° C of THA completely inhibited the ice nucleating abilities of ice nucleating active bacteria or hemolymph protein ice nucleators, but only if the enhancer glycerol was present (Duman, 2002).

While the wax-coated cuticle of insects is generally thought to prevent inoculative freezing, this is not always the case. In late summer, prior to cold adaptation and DAFP production, *D. canadensis* larvae can be frozen by contact with ice at a temperature just 1° C or less below the hemolymph hysteretic freezing point. Throughout the autumn, DAFPs are increased in the hemolymph and these play a role in inhibiting inoculative freezing, along with seasonal modifications of the cuticle, including addition of DAFPs to the epidermal layer underlying the cuticle (Olsen et al., 1998). Once again, the level of inhibition of inoculative freezing by AFPs greatly exceeds the THA present in the hemolymph. One contributing factor to this result is that the measured THA is inversely proportional to the size of the seed ice crystal used in the measurement (Zachariassen

and Husby, 1982; Zachariassen et al., 2002). Because the pores in the cuticle through which ice might propagate are much smaller than the seed crystal used in THA measurements, the protection from inoculation afforded by DAFPs is greater than the THA measured in the hemolymph.

In overwintering *D. canadensis* larvae, DAFPs are present in the hemolymph, gut fluid, and the epidermal cells underlying the cuticle. Tissue specific distributions suggest that certain DAFPs may have evolved to best function in certain sites (Duman et al., 2002). While all of the DAFPs are produced in the fat body, only Group I DAFPs (1, 2, 4, 6) are present in the hemolymph as demonstrated by MALDI-TOF mass spectrometry. However, while none of the Group I DAFPs are present in gut fluid, Groups II (8, 9, 10, 11) and III (3, 5, 7, 12) are found there. Also, consistent with the MALDI-TOF data, only Groups II and III transcripts were identified in midgut epithelia. RT-PCR of epidermal tissue identified DAFPs 4, 6, 8, and 11 (sometimes 1 and/or 2) as the major transcripts. These results suggest that certain DAFPs have evolved to best function in the gut, hemolymph, or epidermal cells.

AFPs and related adaptations (high glycerol, removal of ice nucleators) permit *D. canadensis* larvae to depress their supercooling points, and lower lethal temperatures, to -25°C in northern Indiana. Recent studies with another AFP-producing beetle, *Cucujus clavipes*, demonstrate that AFPs in combination with a suite of additional cold adaptations can extend supercooling to extremely low levels. The latitudinal range of *C. clavipes* is quite broad, from Kentucky to the Arctic, near latitudinal treeline in Alaska. *Cucujus* AFPs (CAFPs) are similar to those of *Dendroides canadensis* beetles that we have studied extensively in Indiana. In fact, some CAFPs are nearly identical to DAFP-1. However, other CAFPs are missing 10 residues near the N-terminus, including three cysteines. This leaves these CAFPs with an odd number of cysteines, therefore precluding the identical disulfide bridging found in the DAFPs (Fig. 3).

Mean supercooling points (SCPs) of both Indiana and Alaska *C. clavipes* populations in summer are $\sim -8^{\circ}\text{C}$, but SCPs of Fairbanks, Alaska, larvae in January 2003 were -42°C while those of Indiana larvae were -24°C . Some individuals collected near Fairbanks had SCPs as low as -57°C , and most larvae from further north, near treeline in the Brooks Range, did not freeze at -64°C . In addition to AFPs, factors that may contribute to the increased supercooling in the Alaskan larvae are higher polyol levels, diapause with greatly reduced metabolic rates, and extensive dehydration. As regards the latter, Alaskan *Cucujus* desiccate from 63.1% body water ($1.70\text{ g H}_2\text{O g}^{-1}\text{ dry wt}$) in August to 35.2%

(0.53 g H₂O g⁻¹ dry wt) in January. While this ~3.2-fold reduction in water volume may cause water stress, it may also promote supercooling by reducing freezable body water and concentrating antifreezes. Since dehydration makes it impossible to get a hemolymph sample for thermal hysteresis measurement in January, hemolymph was taken from cold-acclimated larvae that were not dehydrated and the hemolymph concentrated 3.2-fold, corresponding to the level of dehydration in January larvae. Thermal hysteresis activity (THA) of the hemolymph prior to concentration was 3.29° C (melting point = -3.42° C; freezing point = -6.71° C). However, the THA increased to nearly 13° C following concentration. This is by far the highest THA ever measured.

Freeze Tolerance

While most known AFP-producing animals are freeze avoiding and the AFPs function as antifreezes, several freeze-tolerant insects and centipedes that produce AFPs are known (Tursman and Duman, 1995; Duman, 2001; Duman et al., 2004). The freeze-tolerant centipede *Lithobius forficatus* in northern Indiana has low levels of hemolymph THA in winter (Tursman et al., 1994). AFPs are free in the centipede hemolymph, but they also are present on the surface of cells. Addition of DAFPs, which are immunologically similar to those of the centipede, to *L. forficatus* cells provides protection from damage resulting from freezing (Tursman and Duman, 1995). The mechanism of this protection is unknown. However, AFPs of all types inhibit the potentially damaging process of recrystallization whereby water molecules migrate from the surfaces of ice crystals with higher surface free energies (i.e., smaller crystals with greater radii of curvature) to those with lower surface free energy (i.e., larger crystals) (Knight and Duman, 1986; Tursman et al., 1994). Other cryoprotective mechanisms are likely. Recently, several very freeze-tolerant insects and centipedes from the interior of Alaska were shown to have AFPs (Duman et al., 2004), suggesting that AFPs may also play a cryoprotective role in these organisms.

Applications of Insect AFPs

Because insect AFPs have by far the highest activities of any known AFPs they are excellent candidates for applied uses. One realistic application is in the production of transgenic organisms with increased resistance to freezing. Using the gene for one of the *D. canadensis* AFPs (DAFP-1), we successfully transformed the model plant *Arabidopsis thaliana*, producing plants that decreased their freezing temperatures over controls by 1 to 3° C, depending on how the plants were

cooled (Huang et al., 2002). While this may not seem to provide much protection, note that most late spring and early autumn frost damage results from relatively minor freezes. Attempts to improve this result are ongoing. There are numerous other potential applications for these proteins. For example, insect AFPs might be used to cryopreserve human organs and tissues to allow their storage at subzero temperatures for transplant purposes. Also, the frozen food industry has expressed interest in AFPs because they prevent recrystallization (Knight and Duman, 1986), a source of damage and therefore poor quality, in frozen foods. The beetle AFPs offer the best success for such applications, because they have the highest known antifreeze activities.

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Cardiac Conduction and Resistance to Ventricular Fibrillation in Siberian Hibernator Ground Squirrel *Citellus undulatus*

VADIM V. FEDOROV,¹ RUBIN R. ALIEV,² ALEXEY V. GLUKHOV,¹ ANDREY V. RESNIK,¹ ANDREY ANUFRIEV,³ IRINA A. IVANOVA,¹ OLGA V. NAKIPOVA,⁴ STELLA G. KOLAEVA,^{4*} LEONID V. ROSENSHTRAUKH,¹ AND IGOR R. EFIMOV^{2, 5}

¹ Cardiology Research Center, Moscow, Russia

² Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Puschino, Russia

³ Institute of Biological Problems of Criolithozone, Russian Academy of Sciences, Yakutsk, Russia

⁴ Institute of Cell Biophysics, Russian Academy of Sciences, Puschino, Russia *(passed away in 2003)

⁵ Case Western Reserve University, Cleveland, Ohio, USA

Abstract. Most nonhibernating mammals experience cardiac arrest during hypothermia (15–27° C) due to ventricular fibrillation (VF) or full conduction block. In contrast, hibernators maintain cardiac output even at –2° C to +8° C. Our goal was to assess safety of conduction in the hibernator heart during extreme hypothermia.

We imaged electrical conduction in intact isolated hearts of summer active (n = 5) and winter hibernating (n = 4) ground squirrels *Citellus undulatus* from Siberia at different temperatures varying from +37° C to +2° C. Electrical activity was mapped during normal sinus rhythm and ventricular pacing using CCD camera (500 frames/sec) and voltage-sensitive dye di-4-ANEPPS.

No spontaneous VF was observed in all nine hearts at any temperature. Hearts were able to maintain spontaneous sinus rhythm and normal pattern of epicardial excitation throughout the whole range of studied temperatures. Despite responsiveness to pacing in all hearts, the ventricular conduction velocity was significantly reduced at low temperatures 2–7° C from 80 ± 4 cm/sec to 11 ± 1 cm/sec.

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Our data provides the first direct demonstration that the isolated heart of the ground squirrel *Citellus undulatus* developed ability to maintain normal excitation pattern and protection against spontaneous hypothermia-induced VF in a range of temperatures from +37° C to +2° C.

Introduction

Hypothermia is commonly encountered by emergency physicians. For nonhibernating mammals, including humans, the heart succumbs to cardiac arrest due to full conduction block or VF between 27° and 10° C (Johansson, 1996; Mattu et al., 2002). In contrast, the heart of a hibernator continues to beat at temperatures slightly above freezing point during the hibernating period (Burlington and Milsom, 1989; Wang et al., 2002). VF does not occur either during the onset of hibernation, which is a relatively slow gradual process involving drastic changes in protein expression, or during arousal, which only takes hours and results in increase of protein expression, despite the fact that the body temperature may increase or decrease by over 30° C within one hour (Johansson, 1996). Yet, despite a century of scientific inquiry, the precise mechanisms of protection against hypothermia-induced VF in the hibernator heart are unknown. Duker et al. (1987) proposed that the difference in resistance to hypothermia of the hibernator and nonhibernator hearts is probably mainly due to differences in conduction velocity. This theory is supported by recent observation of significant overexpression of gap junction protein Cx43 in the hibernating myocardium (Saitongdee et al., 2000). However, data on conduction velocity in the intact hibernator heart during hypothermia are practically absent (Ophhof and Rook, 2000).

We studied electrical conduction in hearts of hibernator ground squirrel *Citellus undulatus* from the cold pole region of the Northern hemisphere captured in the Lena river valley of Siberia near city of Yakutsk. The ground squirrels are one of the most resilient hibernators, able to adapt to and spontaneously arouse from core body temperatures as low as -2° to -4° C without freezing (Anufriev and Akhremenko, 1992). The aim of our study was to measure conduction velocity and pattern of excitation at different temperatures ranging from +37° C to +2° C in isolated Langendorff-perfused hearts of summer active (SA) and winter hibernating (WH) ground squirrels.

Material and Methods

Experimental Preparation

Ground squirrels *Citellus undulatus* were trapped in the Lena river valley region of Yakutsk Province and shipped via air to Pushchino, Moscow Province. Five

SA and four WH ground squirrels of either sex were used for the study (see Fig. 1). The SA animals were housed individually at $20 \pm 2^\circ \text{C}$ and 12–12 h light-dark photoperiod before the experiments conducted during June 2003. The average rectal temperature of the SA squirrels was about 37.5°C . To facilitate hibernation in October, when the endogenous cycle of animals reached their hibernating phase, animals were transferred to a darkened cold room ($0 \pm 2^\circ \text{C}$). The WH squirrels had been in more than two successive bouts of hibernation with rectal temperature about 1°C before sacrifice (January 2004). The average weight of the animals was $703 \pm 54 \text{ g}$.

The hearts were isolated and Langendorff-perfused (pressure of $70 \pm 5 \text{ mm Hg}$) with oxygenated (95% oxygen, 5% CO_2) normal Tyrode solution at different temperatures from 37°C to 2°C (Fig. 2). Temperature was gradually reduced by adding ice to the heat exchanger bath and then increased by slow heating the bath. The Tyrode's solution had the following composition (in mmol/L): NaCl 118, CaCl_2 3, MgSO_4 1.1, KCl 4.7, KH_2PO_4 1.2, NaHCO_3 25, and glucose 11, pH was adjusted to 7.35–7.40.

A**B**

Fig. 1. Siberian ground squirrel (*Citellus undulatus*) in summer-active (A) and winter-hibernating (B) states.

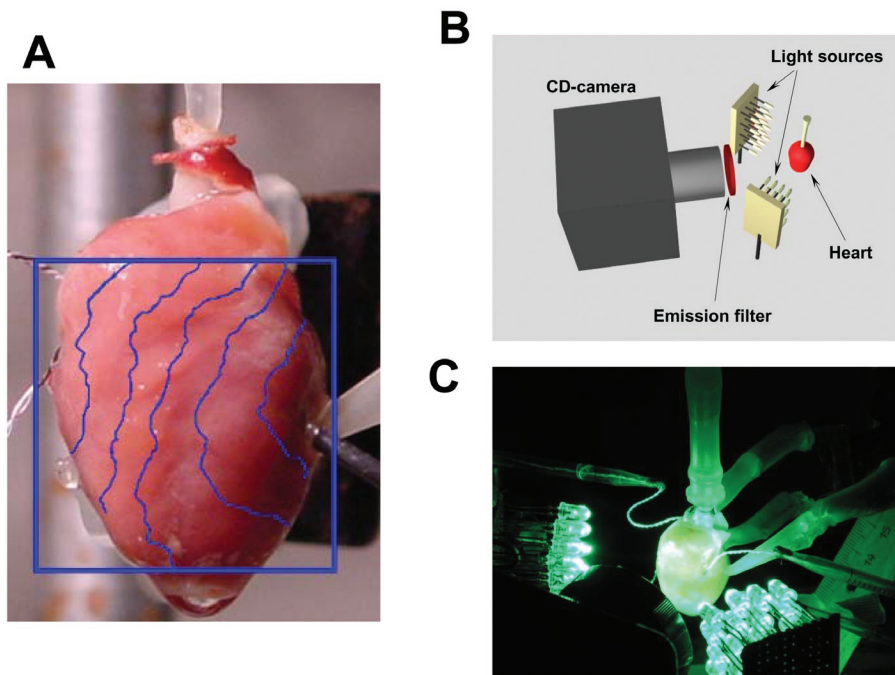


Fig. 2. Experimental preparation and setup.

A. Isolated Langendorff-perfused heart of *Citellus Undulatus*. Optical imaging with voltage-sensitive dye di-4-ANEPPS allowed reconstruction of activation patterns (17x17 mm), which is shown as isochronal map.

B. Imaging was conducted using Dalsa CCD camera at 500 frames/second and 128x127 pixels/frame. Light source consisted of two 4x4 arrays of green light emitting diodes.

C. Photo of the heart and imaging system.

The isolated Langendorff-perfused hearts were equilibrated at 37° C for about 60 min before imaging. After the imaging at normal temperature of 37° C, Tyrode solution was gradually cooled down during 20 min to 25–30° C, 15–20° C, 5–9° C, and 2° C (only for WH), kept at this temperature for at least 10 min for equilibration. At the end of the protocol the heart was rewarmed back to control temperature of 37° C during 30 min. Fluorescent imaging was conducted at every temperature range during spontaneous sinus rhythm (n = 6 hearts) and/or pacing (n = 9 hearts) with basic cycle length of 200–2000 ms.

Electrical activity of the entire ventricular epicardial surface was optically mapped with CCD camera (Dalsa, Canada) with frame rates from 460 to 500

Hz and voltage-sensitive dye di-4-ANEPPS (Molecular Probes, OR). Activation time in each pixel was determined as the time-point of the maximum time derivative of inverted fluorescence intensity $(-dF/dt)_{\max}$ (Salama et al., 1994). Activation maps were reconstructed from obtained matrices of activation times.

Results

Hypothermia (decrease of temperature from 37° C to 2–5° C) did not induce cardiac arrest in any SA and WH studied heart of the Siberian ground squirrel. Neither spontaneous tachyarrhythmia nor asystole were observed in any of the nine experiments during either cooling from 37 to 2° C or rewarming the heart back to 37° C.

Hypothermia did, however, significantly depress spontaneous heart rate. Fig. 3 shows representative optical signals from the center of the field of view of CCD camera at the left ventricle of SA and WH hearts. Traces contain optical action potentials, which are characteristically distorted by contraction-induced movement artifacts that occur during repolarization phase of the cardiac cycle. In most experiments we did not use excitation-contraction uncouplers, often used in optical mapping studies to suppress motion artifacts. We tried to avoid uncouplers due to their known effects on electrical activity. Reduction of temperature resulted in both slowing of the sinus rhythm and increasing delay between excitation and contraction, evident from delay between the upstroke of optical action potential and the movement artifact. Other SA and WH experiments were in agreement with these results. In these isolated hearts ($n = 6$), reduction of temperature from 37 to 2–5° C resulted in reduction of the rate of spontaneous sinus rhythm from 160 ± 13 beats/min to 6 ± 1 beats/min ($p < 0.01$), respectively. Moreover, we observed no evidence of AV block at any temperature in all six hearts in which sinus rhythm was studied. Reduction of temperature from 37° C to 2–5° C resulted in an increase of the AV delay from 71 ± 8 ms to 542 ± 155 ms ($p < 0.01$).

Hearts from both groups maintained qualitatively similar epicardial activation pattern during sinus rhythm at all studied temperatures. Fig. 4 shows data from one of the SA hearts. One can see that despite a significant increase in the conduction time, conduction pattern is preserved. Ventricular epicardial excitation originates as two breakthrough points at the left and right ventricles, with the left ventricular breakthrough slightly preceding the right ventricular breakthrough.

We next determined the effect of temperature on the conduction velocity. Velocity is known to depend on the pacing rate, thus temperature dependence

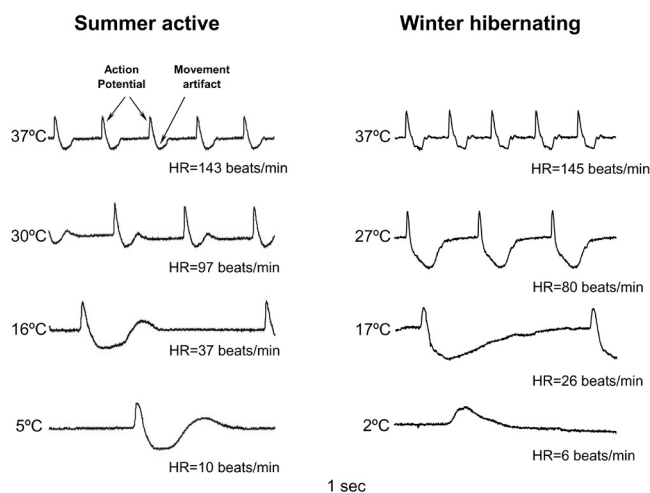


Fig. 3. Optical recordings of electrical activity from the left ventricular epicardium during spontaneous sinus rhythm of summer active and winter hibernating heart at different temperatures.

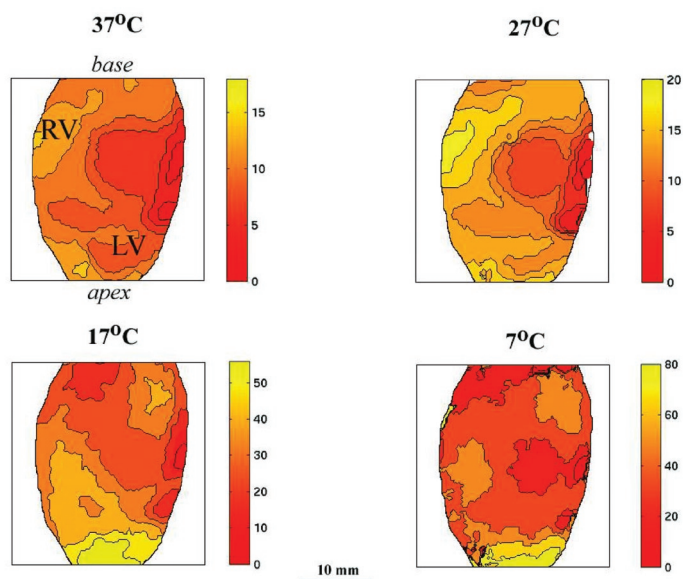


Fig. 4. Pattern of excitation during spontaneous sinus rhythm recorded at different temperatures of the summer active heart. Notice qualitative preservation of pattern of excitation with two sites of breakthrough in the left and right ventricles. Isochrones separated by 2 ms in maps 37°C and 27°C, by 8 ms in 17°C, and by 16 ms in 7°C. RV = right ventricle, LV = left ventricle.

was studied at the same pacing rate(s). However, it was impossible to maintain the same pacing rate throughout the entire range of temperature. For example at high temperatures, the spontaneous sinus node inhibited capture of slow pacing (> 300 ms cycle length). At low temperatures, rapid pacing did not capture (< 500 ms cycle length). Thus, we ablated the sinus node in three SA hearts and used cycle lengths of 1000 and 2000 ms, which resulted in capture at almost any temperature of the protocol.

Fig. 5 presents isochronal maps of activation recorded from the anterior epicardium during left ventricular pacing (1000 ms) at different temperatures. Gradual hypothermia resulted in a gradual directionally heterogeneous slowing of the conduction. At low temperature of 9°C , zones of slow conduction and conduction block developed near the septum and at the right ventricular epicardium. Normal conduction pattern and velocity were fully restored after rewarming of the preparation (see map at 37°C).

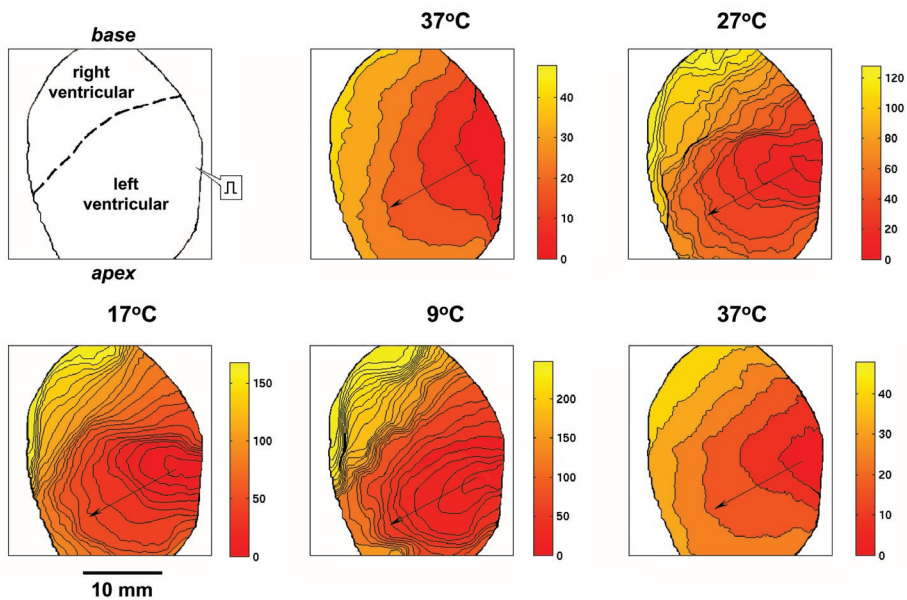


Fig. 5. Isochronal maps of ventricular activation of isolated ground squirrel heart at various temperatures during pacing (1000 mc). Shown are the schematic presentation of the field of view from which the fluorescent signals were recorded. The five isochronal maps (8 ms apart) of the anterior epicardial activation are shown. They were recorded during control temperature (37°C), cooling (27°C , 17°C , and 9°C) and after subsequent rewarming back to 37°C .

Due to possible block of conduction in the right ventricle, we measured conduction velocity during reduction of temperature only in the left ventricle. Arrows in Fig. 5 show the direction of conduction velocity measurements.

Fig. 6 summarizes conduction velocity data from all five SA and four WH hearts studied. Conduction velocity significantly decreased from 71 ± 4 to 14 ± 1 cm/sec and from 88 ± 12 to 9 ± 1 cm/sec with decrease of temperature from 37°C to $5\text{--}9^\circ\text{C}$ (SA) and to 2°C (WH), respectively. Exponential fits describe these relationships well ($r^2 = 0.97$ and 0.93 for SA and for WH, respectively). We observed difference in conduction velocity of SA and WH hearts at normal temperature. However, these differences did not reach statistical significance perhaps due to small sample size. At low temperatures, conduction velocity was the same in both states of the myocardium.

Finally, we investigated optical action potential morphology without motion artifact. We repeated the experimental protocol using excitation-contraction uncoupler (BDM, 15 mM) in four WH intact Langendorff-perfused hearts (Cheng et al., 2004). Then, after the end of protocol, we isolated the papillary muscles from four WH hearts and obtained microelectrode recordings of action

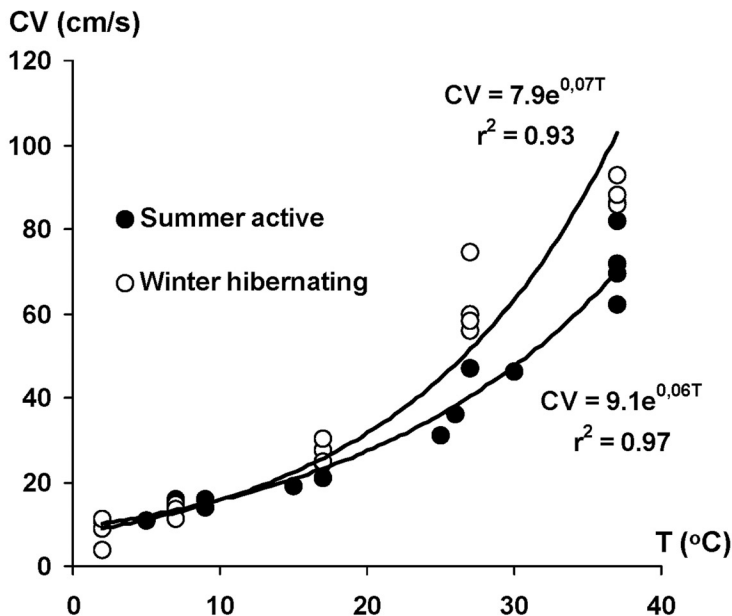


Fig. 6. The dependence of ventricular conduction velocity from temperature. Data summarizes five summer active and four winter hibernating experiments.

potentials at different temperatures. Fig. 7 shows a series of representative optical records from the center of right ventricular (A) and microelectrode records (B) from the papillary muscle of the same WH heart at different temperatures. Reduction of temperature from 37° C to 7° C resulted in significant decrease of the depolarization rate during action potential upstroke and in significant increase of the action potential duration at 90% repolarization level in both experiments from 128 ms and 112 ms to 710 and 750 ms, respectively. Another WH experiment yielded similar results. Optical and microelectrode recordings were not significantly different during recordings at all temperatures. Table 1 summarizes findings from microelectrode recording, which are consistent with data of other groups (Liu et al., 1987).

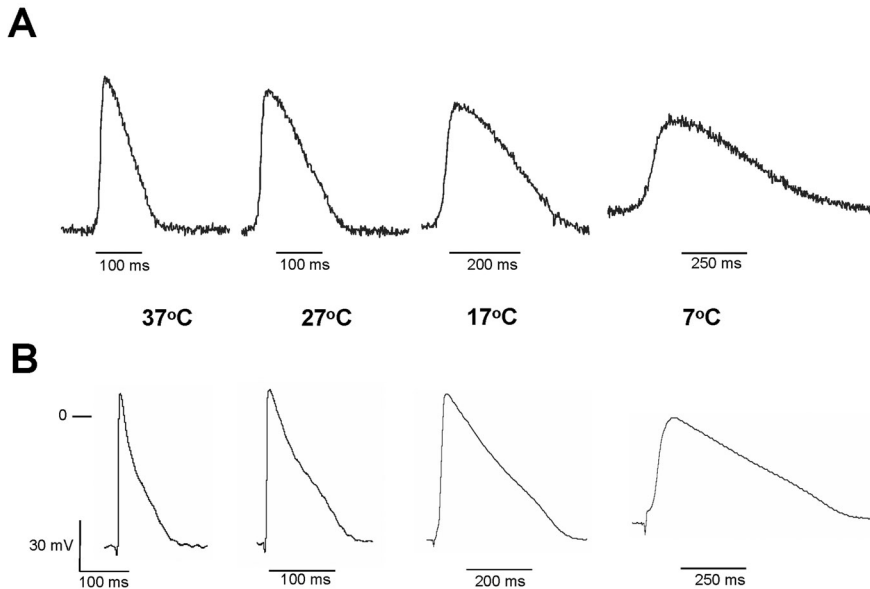


Fig. 7. Optical (A) and microelectrode (B) recordings of action potentials from ventricular myocardium of winter hibernating heart at different temperature. 2, 3-butanedione monoxime was used to suppress mechanical artifacts in both cases. Optical recordings were obtained from the right ventricular epicardium (A). Microelectrode recordings were subsequently obtained from papillary muscles (B) of the same heart.

Table 1. Action potential amplitude and resting potential.

Temperature (° C)	37	27	17	7
Resting potential (mV)	-81 ± 3	-80 ± 1	-73 ± 2	-58 ± 5
AP amplitude (mV)	89 ± 1	95 ± 3	93 ± 7	58 ± 16

Discussion

The Heart Rate in Hibernation

The heart in hibernation has been a subject of investigation for well over a century. In 1832 Hall (cited in Dave and Morrison, 1955) suggested that hibernators are able to maintain cardiac output during hibernation at extremely low temperatures due to hyperexcitability of their heart muscle as compared to the heart of the nonhibernating mammals. In more recent publications, the heart rate of different hibernators was shown to decrease to 2–10 beats/minute at temperature 2–8° during hibernation (*in vivo*) or hypothermia (*in vitro*) (Dave and Morrison, 1955; Burlington and Darvish, 1988). Contractile function of the hibernator heart is maintained during artificial cooling *in vitro*, in deep hypothermia, and during rewarming. In contrast, the human heart shows a limited tolerance to cold, which results in life-threatening conditions of accidental hypothermia victims (Johansson, 1996; Mattu et al., 2002). Similar problems have been observed in other nonhibernators during experimental hypothermia (Burlington and Milsom, 1989). Experimental studies have shown that the isolated perfused heart of mammalian hibernators maintains circulation at 0–7° C, whereas hearts isolated from nonhibernating mammals succumb to arrhythmia at 30–16° C and usually experiences cardiac arrest at 16–10° C (Burlington and Darvish, 1988; Johansson, 1996).

Our data for the first time presented conduction maps of hibernator's heart *Citellus undulatus* at low temperature, which show remarkable preservation of excitation pattern during sinus rhythm at different temperatures ranging from 37° to 2–5° C, despite nearly 20-fold slowing of the heart rate (Fig. 3–4).

Ventricular Fibrillation Resistance at Low Temperature

Mechanisms of hibernator resistance to spontaneous hypothermia-induced VF remain unclear. The differences in propensity to VF between hibernator and non-hibernator could be explained by the reentrant theory of VF. Indeed, non-hibernators develop a great degree of conduction and repolarization heterogeneity during moderate hypothermia (Salama et al., 1998). Activation and repolar-

ization heterogeneity is a likely trigger of unidirectional conduction block that could lead to onset of VF in nonhibernators. In contrast, our data for the first time demonstrates that hibernator *Citellus undulatus* preserves nearly normal, uniform conduction and repolarization pattern even at extreme hypothermia (Fig. 4–7). In none of five SA and four WH studied hearts was spontaneous tachyarrhythmia observed during gradual decrease of temperature from 37° to 2° C and during gradual increase back to 37° C.

Duker et al. (1987) proposed a theory that the difference in susceptibility to VF is probably due to differences in conduction velocity. However, data on conduction velocity in intact heart at low temperature are very sparse. Our report presents the first direct measurements of conduction pattern and velocity of hibernator heart *in vitro*. We demonstrated seven-fold decrease in the conduction velocity during reduction of temperature from 37° to 2–9° C in both SA and WH hearts. Our direct measurements agree with ECG data presented by Dave and Morrison (1955), who showed that QRS interval of three hibernator species during hibernation (body temperature 0.5°–9° C) increased by 5–10 fold.

Molecular mechanisms of protection against asystole and/or VF during hypothermia remain unclear. Several mechanisms could be involved. Impaired Na⁺ and Ca²⁺ channel function and decreased gap junctional conductance could contribute to slow conduction (Shaw and Rudy, 1997), which is a key factor of reentrant arrhythmogenesis. Liu et al. (1991) showed that in hedgehog cardiac preparations, peak of Ca²⁺ current was not significantly affected by hypothermia. On the other hand, the Na⁺ current in hedgehog was less influenced by hypothermia than in rat (Liu et al., 1991). These observations suggest that both Na⁺ and Ca²⁺ currents may contribute to the hypothermia tolerance and resistance to VF during hypothermia in mammalian hibernators. Saitongdee et al. (2000) suggested that Cx43 gap junctional channels overexpression in the ventricle of hibernator (hamster) may represent a compensatory response in order to maintain sufficient intercellular gap junction communication during physiological conditions that would otherwise reduce conductance. Thus, altered function and/or level of expression of Na⁺, Ca²⁺, and Cx43 channels could contribute to resistance of hibernator heart to conduction block and/or spontaneous VF during hypothermia.

Seasonal Differences of Hibernators in Arrhythmia Resistance

Duker et al. (1983) demonstrated that arrhythmia resistance of the hibernator heart is seasonally dependent. The woodchuck hearts during winter were

completely resistant to VF, but were vulnerable to VF during summer. However, in other mammalian hibernators, the heart resistance to cold is season-independent (Burlington and Milsom, 1989). No differences in action potential associated with hypothermia were noted in WH and SA hedgehog muscle fibers (Liu et al., 1987). In our study we used both SA and WH animals. We didn't observed seasonal dependence in the heart resistance to hypothermia. However, we used of a limited number of hearts ($n = 9$).

Conclusions

Our data from Siberian ground squirrel *Citellus undulatus* demonstrated maintenance of excitation pattern of normal sinus rhythm during hypothermia and lack of spontaneous VF in SA and WH hearts at temperature range from 37° C to 2° C. Future studies will target molecular mechanisms of hypothermia resistance, which are likely due to changes in function and expression of sodium, calcium, and gap junctional channels as suggested by dependence in morphology of action potentials with temperature in our preparations.

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The Correlation Between Akt Activity and Hibernation

DECHENG CAI,¹ RICHARD M. MCCARRON,² DONNA SIECKMANN,² AND JOHN M. HALLENBECK¹

¹Stroke Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

²Resuscitative Medicine Department, Naval Medical Research Center, Silver Spring, MD 20910-7500, USA

Abstract. Mammalian hibernation is an evolutionary adaptation to overcome unfavorable environmental conditions, such as cold and starvation, by reducing core body temperature and metabolism. Akt is a phosphatidylinositol-3 kinase-regulated serine/threonine protein kinase in the insulin/insulin-like growth factor signal transduction pathway, which plays a critical role in the balance between survival and apoptosis. We studied whether Akt phosphorylation is involved in the regulation of mammalian hibernation. Our results show that S473 phosphorylation of Akt in brain and muscle was significantly reduced, as was its kinase activity, in the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) during hibernation. The data presented here raise the possibility that down-regulation of Akt phosphorylation plays a regulatory role in hibernation.

Introduction

Mammalian hibernation is an evolutionary adaptation to cold weather and starvation. Body temperature decreases from about 37° C to ~5–6° C are associated with profound reductions of blood flow and oxygen delivery in the brain (Frerichs et al., 1994). The reduction of blood flow during hibernation has no adverse effect on hibernators but would lead to rapid autolysis of brain tissue in the absence of adjustments that characterize this tolerant state (Astrup et al., 1981). We have been interested in hibernation as a model to study natural

Barnes BM, Carey HV, eds (2004) *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium*. Biological Papers of the University of Alaska, number 27. Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska.

tolerance to brain ischemia. Understanding the regulation of hibernation could guide researchers toward effective therapeutic strategies for stroke and brain trauma.

In the nematode *C. elegans*, an insulin/insulin-like growth factor 1 (IGF-1) signaling pathway regulates metabolism, development, and longevity (Kimura et al., 1997; Morris et al., 1996). DAF-2, an insulin/IGF-1 receptor, activates AGE-1, a phosphatidylinositol-3 kinase (PI3K) that leads to activation of Akt-1 and Akt-2, which are Akt/PKB homologs. Akt-1 and Akt-2 phosphorylate DAF-16, a forkhead transcription factor with mammalian orthologs, FoxO1 (FKHR), FoxO3a (FKHRL1), and FoxO4 (AFX) (Burgering and Kops, 2002). This ultimately results in inhibition of DAF-16 regulated transcription.

Hibernation in mammals, which shows some physiological similarity with dauer larva formation in *C. elegans*, is also a metabolically arrested stage allowing survival under unfavorable environmental conditions. During hibernation, insulin-like growth factor serum concentrations and bioactivity are markedly reduced (Schmidt and Kelley, 2001). Here we examined whether the insulin/IGF-1 signaling pathway is involved in the regulation of hibernation in mammals by studying a key step in signal transduction pathway, Akt phosphorylation, during hibernation. The experiments reveal that hibernation in the 13-lined ground squirrel is associated with down-regulation of S473-phosphorylation of the serine-threonine protein kinase, Akt.

Materials and Methods

Animals and Induction of Hibernation

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were used in this study. Induction of hibernation in ground squirrels was carried out as previously described (Frerichs et al., 1994). Briefly, the squirrels were housed individually in a room with an ambient temperature of 21° C and a 12:12-hour light-dark cycle and were fed standard rodent diet. The squirrels were placed in a dark and cold chamber that was kept at 4° C and 60% humidity. The animals labeled as hibernators were studied after two to four days of hibernation (H group) with the body temperature of ~5–7° C. Animals that were maintained in the holding room and displayed a low and stable body weight and did not show any signs of torpor or hibernation, with the body temperature of ~36–38° C, were referred to as winter warm-adapted, active (A group) animals. Samples (a cerebral hemisphere and sections of femoral [skeletal] muscle) of both active and hibernating groups were collected on the same day between November and March. The tis-

sues were flash frozen in liquid nitrogen, and stored in a freezer at -70°C until analysis.

The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and were approved by the NIH Institutional Animal Care and Use Committee.

Western Blot Analysis

Protein extracts were prepared from the brains and muscles of active and hibernating ground squirrels. Proteins (75 μg) were separated on 10% tris-glycine SDS polyacrylamide gel, then transferred to PVDF membranes. Samples were incubated with 1:1000 anti-Akt antibody or 1:1000 anti-phospho-Akt antibody raised against S473-phosphorylated Akt (Cell Signaling Technology, Beverly, MA). Blots were developed with an ECL Western blotting analysis system and exposed to Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Akt Kinase Assay

An Akt Kinase Assay Kit (Cell Signaling Technology) was used according to the manufacturer's instructions. Briefly, brain tissues from active and hibernating animals were lysed under native conditions. Samples were suspended in 30 μl of kinase buffer with 200 μM ATP and 1 μg GSK-3 fusion protein and incubated for 30 min at 30°C . The reaction was terminated with 30 μl 3x SDS sample buffer and boiling for 5 min. A 20 μl sample aliquot was separated by SDS-PAGE. Bands in the Western blot were detected by anti-phospho-GSK-3 antibody.

Image Quantitative and Statistical Analysis

Western blot image was quantified by scanning with a densitometer and analyzed with NIH image software. Data are expressed as mean \pm SD. Data were statistically analyzed with an unpaired Student's t test. Differences were considered significant at $p < 0.05$.

Results and Discussion

Down Regulation of Phosphorylation of Akt in Hibernating Ground Squirrel

We compared total and phosphorylated Akt levels in brain and muscle from active and hibernating ground squirrels. Western blots of S473-phosphorylated

Akt indicated a remarkable decrease of Akt phosphorylation in both brain and muscle tissue obtained from hibernating ground squirrels compared with active animals (Table 1). Total Akt expression levels in either tissue did not differ between the two states.

*Table 1. Down-regulation of S473-phosphorylation of Akt in the thirteen-lined ground squirrel during hibernation. Tissue extracts from brains and muscles of active and hibernating thirteen-lined ground squirrels were separated with SDS-PAGE. Data represents the optical density of scanned Western blot image (n = 4 in each group; * p < 0.001).*

		Akt expression	
		Active	Hibernating
Total Akt	Brain	80.7 ± 2.0	76.0 ± 3.6
	Muscle	58.1 ± 20.0	67.4 ± 20.9
p-Akt (Ser 473)	Brain	51.0 ± 9.0	19.2 ± 3.5*
	Muscle	108.3 ± 13.9	48.0 ± 10.8*

Decreased Akt Kinase Activity in Hibernating Ground Squirrels

The *in vitro* assay of Akt kinase activity showed a significant ($p = 0.023$) decrease in Akt kinase activity (measured as optical density units) in brain samples from hibernating ground squirrels versus active controls (Table 2).

*Table 2. Akt kinase assay. Brain tissue extracts from active and hibernating animals were immunoprecipitated with anti-total Akt antibody as described in Methods. Data represent the optical density of scanned Western blot image (n = 3; * p = 0.023).*

Akt kinase activity	
Active	Hibernating
110 ± 23.3	64.5 ± 19.3*

The present data indicate that during hibernation, levels of Akt phosphorylation in brain and muscle are significantly decreased while total Akt levels remain unchanged. The hypophosphorylation of Akt noted during hibernation

corresponds with a reduction of brain Akt kinase activity to about one-half the level seen in active ground squirrels. Recent reports indicate that the differential expression of Akt also occurs during hibernation in other animals (Lee et al., 2002; Eddy and Storey, 2003; Hoehn et al., 2004). Akt that is activated by phosphorylation is well established as a critical component of survival signaling, particularly in trophic factor-mediated survival (Datta et al., 1999), but also in states of ischemic tolerance induced in brain (Yano et al., 2001). Activated Akt inhibits apoptosis by phosphorylating Bad, a pro-apoptotic member of the Bcl-2 family (Datta et al., 1997). This mechanism also prevents Bad-mediated activation of the pro-apoptotic factor, caspase 9, to further suppress apoptosis (Cardone et al., 1998). Phosphorylation of forkhead family members, FoxO1, FoxO3a, and FoxO4, which are Akt substrates, interferes with DNA binding of these transcription factors and leads to their binding to 14-3-3 proteins in the cytoplasm (Brunet et al., 1999; Datta et al., 1999). Phosphorylated forkhead proteins are thereby prevented from inducing transcription of FasL, resulting in decreased activation of caspase 8 and thereby further suppressing apoptosis (Juo et al., 1998). Conversely, studies indicating a survival-promoting role for hypophosphorylated Akt in dauer larva arrest in *C. elegans* have been recently extended to mammalian cells. When Akt phosphorylation is antagonized by inhibition of the PI3K pathway and becomes hypophosphorylated, FoxO transcription factors can upregulate expression of the free radical scavengers, cytosolic catalase, and manganese superoxide dismutase (Taub et al., 1999; Kops et al., 2002a). Transcriptional upregulation of the growth arrest and DNA damage response gene, Gadd45a, also occurs (Tran et al., 2002). Transcriptionally active FoxO proteins also regulate cell cycle progression and can cause arrest at the G1/S and G2/M checkpoints (Tran et al., 2002). Transcription of the cyclin-dependent kinase inhibitor, p27^{kip1}, and a member of the retinoblastoma family of nuclear pocket proteins, pRb2/p130, are involved in this cell cycle arrest that leads to the reversible state of quiescence (Kops et al., 2002b) (Fig. 1).

Taken together the evidence indicates that the insulin/IGF-1 signaling pathway can use different mechanisms to promote cellular tolerance to stressful conditions with Akt in either the active (phosphorylated) or inactive (dephosphorylated) states. Hibernation is a state in which mammals become tolerant to potentially lethal reduction of brain blood flow and oxygen delivery for protracted periods of time. The induction or maintenance of this physiologic state appear to involve an inhibition of Akt activity similar to that known to occur in the dauer larva program in *C. elegans*. A screen of genetic mutants in *C. elegans* has

identified reduced activity of the insulin/insulin-like growth factor pathway as a regulator of high-level resistance to hypoxia in these animals (Scott et al., 2002). Future research may lead to more precise identification of role(s) of Akt activity in hibernation and the characterization of mechanisms associated with ischemic tolerance.

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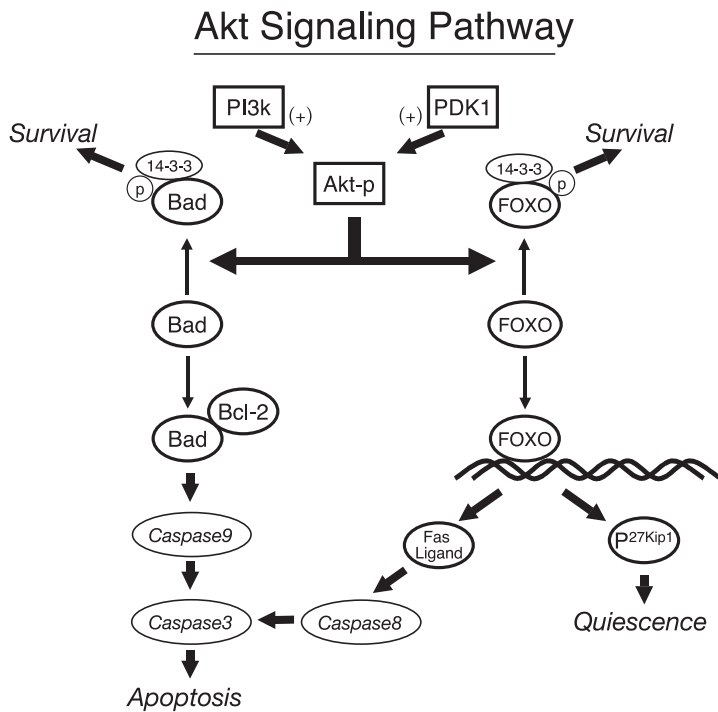


Fig. 1. Akt signaling pathways. Akt regulates cell fate leading to survival, apoptosis or quiescence through the phosphorylation of its target gene Bad and FOXO.

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Protection from Traumatic Brain Injury During Hibernation

KELLY L. DREW,¹ FANG ZHOU,¹ XIONGWEI ZHU,² RUDY J. CASTELLANI,²
AND MARK A. SMITH²

¹ Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska, USA

² Institute of Pathology, Case Western Reserve University, Cleveland, Ohio, USA

Abstract. The purpose of this study was to determine if hibernating brain tissue is tolerant to penetrating brain injury modeled by insertion of microdialysis probes. Guide cannulae were surgically implanted in striatum of arctic ground squirrels before any of the animals began to hibernate. Microdialysis probes were then inserted in arctic ground squirrels during hibernation and in others during euthermia. Brain tissue from hibernating and euthermic arctic ground squirrels was examined three days after insertion of microdialysis probes. While extracellular concentrations of glutamate increased similarly in both groups of animals, tissue response, indicated by examination of H&E stained tissue sections and immunocytochemical identification of activated microglia, astrocytes, and hemoxygenase-1 immunoreactivity, was dramatically decreased around probe tracks in hibernating compared to euthermic controls. No difference in tissue response around guide cannulae was observed between groups. Further study of the mechanisms underlying neuroprotective aspects of hibernation may lead to novel therapeutic strategies for stroke and traumatic brain injury.

Introduction

Hibernation is known best for suppression of metabolism and body temperature. Less well recognized are the numerous, potentially neuroprotective aspects of hibernation physiology. Heterothermic mammals tolerate a variety of central nervous system insults including hypoxia (Bullard et al., 1960; D'Alecy et al.,

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1990) and oxygen glucose deprivation better than homeothermic mammals. *In vitro* studies suggest tolerance is enhanced during torpor and at cold temperatures (Frerichs and Hallenbeck, 1998). Here we demonstrate neuroprotection using an *in vivo* model of traumatic brain injury in the hibernating state. Better understanding of mechanisms of neuroprotection in heterothermic mammals has potential to lead to development of novel therapies and improved prognosis for brain-injured patients.

Microdialysis is an accepted method for extracting analytes of interest from the extracellular space in freely moving animals. The technique has proven effective in numerous applications where correlations in neurotransmitter release and behavior are verified by pharmacological manipulations (Porkka-Heiskanen et al., 1997; Tanda et al., 1997). Nonetheless, insertion of guide cannulae and microdialysis probes into brain tissue produce stab-like wounds characteristic of traumatic brain injury with associated release of IL-1 β , gliosis infiltration of granulocytes and neuronal degeneration (Woodrooffe et al., 1991; Benveniste and Diemer, 1987; de Lange, 1995; Clapp-Lilly et al., 1999). In the present study we assessed tissue pathology around microdialysis probes and guide cannulae in euthermic and hibernating arctic ground squirrels to test the hypothesis that hibernation attenuates the post-traumatic tissue response seen with penetrating brain injury. Results show marked differences in tissue response in hibernating versus euthermic ground squirrels.

Methods

Surgery

All procedures were approved by the Institutional Animal Care and Use Committee. Detailed methods are described in Zhou et al. (2001). Briefly, guide cannulae were implanted bilaterally in euthermic arctic ground squirrels (*Spermophilus parryii*) weighing 650–840 g at the time of surgery. Telemetry transmitters (model VM-FH, Minimitter, OR) used to monitor core body temperature were implanted intraperitoneally. Guide cannulae (CMA, Acton, MA) were stereotactically positioned above the right and left striatum (AP = 13.5 or 14 mm, L = \pm 3.25 mm, D = $-$ 4.0 mm) described in detail by Osborne et al. (1999). Animals housed at 20–22° C; 12:12 hour light:dark, during ten days post-op recovery were transferred to the cold chamber with an ambient temperature of 2–4° C and a 4:20 hour light:dark cycle. Body temperature was monitored at least one day prior to and throughout the microdialysis experiment.

Microdialysis

Microdialysis procedures and experimental protocol were the same as described in detail by Osborne et al. (1999), except that in the present study, all components of the microdialysis system were sterilized via heat (autoclave), ethylene oxide (Anprolene, Andersen Products, Haw River, NC) or 0.2 μm filtration (Acrodisc, Pall Corporation, Ann Arbor, MI). Microdialysis probes (CMA 12/04, O.D. = 0.5 mm) were slowly inserted into the right and left striatum through guide cannulae while animals were hibernating or euthermic. Euthermic animals were lightly anesthetized with halothane, induced as described for surgery, maintained at 1% for approximately 5 minutes. Euthermic animals recovered from anesthesia within about 15 min of inserting probes. Microdialysis probes were perfused with artificial cerebrospinal fluid as described in Zhou et al. (2002). All microdialysis experiments were performed in the hibernaculum with an ambient temperature of 2–4° C.

Dialysate collected at 0.6 $\mu\text{l}/\text{min}$ during the day or 0.1 $\mu\text{l}/\text{min}$ overnight was analyzed for glutamate by HPLC with fluorescence detection as described by Zhou et al. (2002). Total white blood cell counts were performed to assess leukocytopenia during hibernation and to monitor evidence of infection.

Tissue Preparation

After three days of microdialysis sampling, arctic ground squirrels were euthanized and brains collected for analysis as described by Zhou et al. (2001).

Histology

Prior to paraffin embedding, the whole brain was trimmed, perpendicular to the probe track, into three parts similar in thickness. Each part of brain tissue was embedded in paraffin, and 6 μm thick, consecutive sections were prepared on a sliding microtome for further analysis. Hematoxylin and eosin (H&E) staining was performed on sections around probes and guide cannulae of hibernating ($n = 4$ squirrels) and euthermic ($n = 3$ squirrels).

Immunocytochemistry (ICC)

Brain tissue sections were prepared as described by Zhou et al. (2001) and incubated overnight at 4° C with either (1) immunoaffinity purified rabbit polyclonal antibody against GFAP (1:1000) (glial fibrillary acidic protein, kind gift from Dr. Gambetti), used as an established marker for reactive astrocytes; (2) biotinylated RCA-1(1:300) (Vector Laboratories, Inc., Burlingame, CA) that

recognizes microglia; or (3) immunoaffinity purified rabbit polyclonal antibody against hemeoxygenase (HO-1; Stressgen Biotechnologies Corporation, Inc., Victoria, BC, Canada), which is used as a marker of oxidative stress. Sections stained by GFAP and HO-1 were then incubated in goat anti-rabbit (ICN, Costa Mesa, CA) antisera for 30 minutes followed by rabbit-specific peroxidase-antiperoxidase complex for 1 hour (Sternberger Monoclonals, Inc., and ICN, Cappel); and the staining was developed using 3,3'-diaminobenzidine (DAB; DAKO Corp., Carpinteria, CA). Sections treated with RCA-1 were incubated with avidin D peroxidase (Vector Laboratories, Inc., Burlingame, CA) for 1 hour and then developed by DAB. The sections were then dehydrated through ascending ethanol and xylene solutions for mounting.

Statistics

Effects of surgery and hibernation state on leukocyte counts, obtained on the last day of dialysis, were determined using a 2x2 ANOVA design and Tukey post-hoc comparisons. Within-animal comparisons across time for leukocyte counts in euthermic animals and dialysate concentrations of glutamate in euthermic and hibernating animals were made using a repeated measures ANOVA design (SAS for Windows, Version 8. SAS Institute Inc., Cary, NC).

Results

At the time probes were inserted, mean body temperature (\pm SD, $n = 3-4$) of euthermic arctic ground squirrels was ($35.9 \pm 1.6^\circ\text{C}$) and for hibernating arctic ground squirrels was ($4.1 \pm 0.8^\circ\text{C}$). Body temperature of hibernating animals remained low (less than 5°C) throughout the dialysis sampling period. The concentration of glutamate in the dialysate increased initially to the same extent in both groups, stabilized by day two, and remained stable over three days of dialysis (Fig. 1). Two of four hibernating squirrels remained in torpor with no appreciable increase in body temperature between probe implantation and euthanasia. Insertion of probes induced arousal in the other two hibernating squirrels. One of these animals re-entered torpor within 24 hours and the other remained euthermic except for a brief dip in body temperature on day two, where T_b decreased to 21.2°C and again on day three where T_b was 27.5°C at the time of euthanasia.

Microdialysis probe and guide cannulae tracks were easily distinguished based on position (depth) of placement and a clear difference in track diameter. Examination of H&E stained tissue sections indicated an obvious response around the guide cannula in all hibernating and euthermic animals, with no

convincing difference among the cases. However, results from the sections with probe tracks showed a clear difference in tissue reaction between hibernating and euthermic animals (Fig. 2). In the hibernating animals there was simply a small space surrounded by slightly pale neural parenchyma. Routine light microscopy showed no other cellular tissue response around the probe track. By contrast, euthermic animals showed a number of histological changes, including reactive astrocytosis, macrophage infiltrate, and axonal swellings. Based on examination of H&E stained sections, one hibernating animal that demonstrated a brief interbout euthermic period was classified as hibernation-like, while the other animal that aroused after probes were inserted and remained euthermic until euthanasia was classified as euthermic-like.

Activated microglia, identified using RCA-1 binding, were seen around the probe tract in euthermic tissue ($n = 3$ squirrels; six striata, not shown). In contrast, the probe tract was barely discernable in hibernating tissue ($n = 2$ squirrels; four striata, not shown). While resting, ramified microglia were seen throughout the hibernating tissue section; no activated microglia were present around the probe tract. Astrocytosis, also visible in H&E stained sections (Fig. 2), was confirmed using antibodies against GFAP. GFAP immunoreactivity was more intense around the probe tracts in euthermic animals compared to hibernating animals (not shown). HO-1 immunoreactivity was present around the probe tract in euthermic tissue (Fig. 3A). In contrast, HO-1 immunoreactivity was absent in hibernating tissue (Fig. 3B). Tissue response following acute injury experienced during hibernation was indistinguishable from tissue response in euthermic squirrels in one animal that aroused immediately after injury. In contrast, tissue response in hibernating squirrels that re-entered torpor after a brief period of euthermia was similar to other hibernating animals except for an increase in GFAP immunoreactivity.

Leukocytes in whole blood decreased from $6,357 \pm 2,739/\text{mm}^3$ in euthermic animals to $456 \pm 98/\text{mm}^3$ in hibernating animals, (mean \pm SD, $n = 8-10$, $p < 0.0001$ main effect of state). Leukocyte counts were similar in the operated groups compared to unoperated control animals, and counts on the day of surgery and the first and last day of the experiment in euthermic animals remained stable (mean \pm SD; day of surgery, $7,583/\text{mm}^3 \pm 1,443/\text{mm}^3$; day one of dialysis, $8,812/\text{mm}^3 \pm 3,071/\text{mm}^3$; last day of dialysis $7,343/\text{mm}^3 \pm 3,360/\text{mm}^3$). Similar leukocyte counts in operated and unoperated animals and stability of counts during the duration of experimental protocol were consistent with clinical observations that operated animals were free of infection.

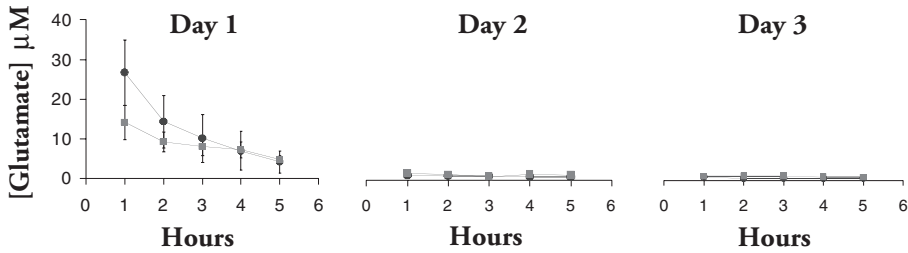


Fig. 1. Insertion of microdialysis probes through indwelling guide cannulae produced an initial increase in extracellular glutamate, indicative of excitotoxicity seen in most types of central nervous system injury. Increase in glutamate did not differ between hibernating (circles) and euthermic (squares) squirrels. Extracellular glutamate concentrations decreased and stabilized in both groups by days two and three.

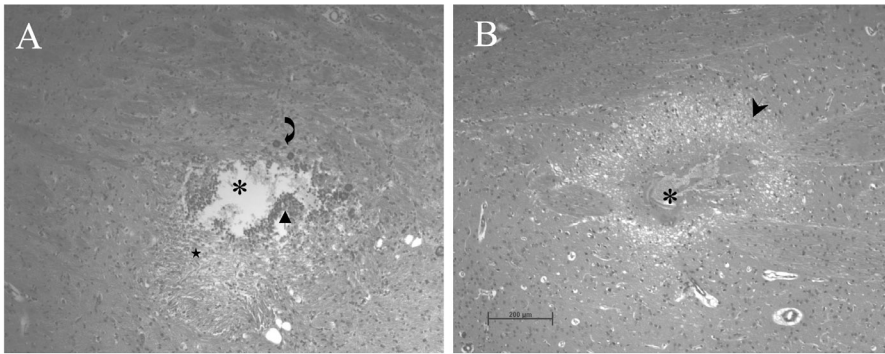


Fig. 2. A greater tissue reaction is seen in H&E stained sections in euthermic animals (A) as compared with hibernating animals (B). Asterisks (*) identifies the probe track. Section from euthermic animal (A) demonstrates a probe cavity, accompanied by mononuclear inflammatory infiltrate (arrow), axonal swellings (curved arrow) and fibrillary gliosis (astrocytosis) (star). In contrast, brain from hibernating animal (B) shows the probe cavity with no discernible inflammatory or reparative reaction. Specifically, no macrophages are seen, and there are no swollen axons or histological evidence of astrocytosis. There is simply a cavity surrounded by slight pallor of the surrounding neural parenchyma (arrow head). Scale bar is 200 μ m.

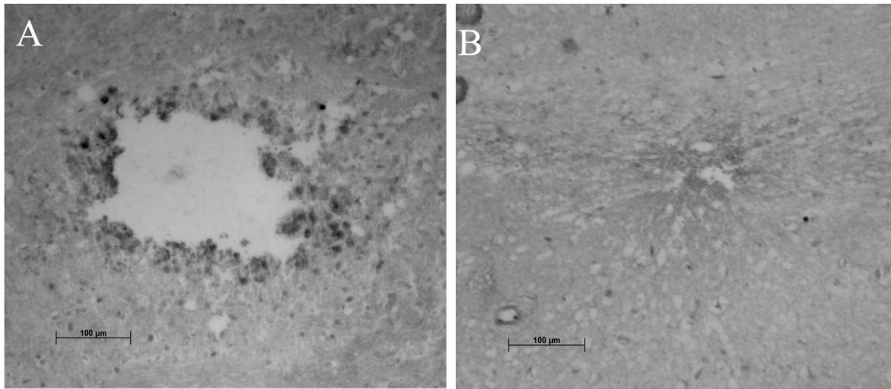


Fig. 3. Immunoreactivity to hemeoxygenase-1 (HO-1), a protein induced by oxidative stress, is increased around the probe tract in euthermic squirrels (A) but is absent in hibernating squirrels (B). Scale bar is 100 μm.

Discussion

Studies show that following central nervous system trauma, such as acute ischemic stroke, cardiac arrest, or traumatic brain injury, neuronal damage continues to progress after the initial insult (McIntosh et al., 1998). Extracellular glutamate increases early in the neurodegenerative cascade, which can begin with disruption of energy balance when oxygen and nutrient delivery fail to meet demand and with mechanical disruption of cellular integrity in the case of penetrating brain injury. Exhaustion of energy stores rapidly leads to loss of ion homeostasis, causing an influx of Na^+ and Cl^- ions, edema, neuronal depolarization, release of glutamate, and opening of voltage gated ion channels, including voltage gated calcium channels. Increased glutamate in the extracellular space further stimulates Ca^{2+} influx into neurons. Subsequent generation of reactive oxygen species as well as other events leads to both necrotic and apoptotic cell death (Dirnagl et al., 1999; Arundine and Tymianski, 2004). In addition, activation and nuclear translocation of stress-activated protein kinases, NF- κ B, and other transcription factors initiates a pro-inflammatory reaction. Intervention at any point of the cytotoxic cascade has potential to minimize brain damage. While glutamate overflow follows a similar time course in both hibernating and euthermic squirrels, increasing initially and then decreasing and stabilizing over time, evidence of neuronal cell death, oxidative stress, and inflammatory response is dramatically decreased in hibernating brain. Attenuation of the cyto-

toxic cascade in hibernating ground squirrels downstream to glutamate is consistent with down-regulation of glutamate receptors and/or enhanced Ca^{2+} homeostasis. This, coupled with immune modification such as the dramatic decrease in circulating leukocytes as well as hypothermia, likely contribute to suppressed tissue response in hibernating squirrels. Observations in animals that remained in torpor for variable lengths of time during the three-day post-injury period suggest the tissue response is delayed as much as it is attenuated. Further quantitative analysis of volume of damaged tissue using larger sample sizes is necessary to distinguish attenuation from delay. Even modest decreases in brain temperature attenuate the extent of ischemic neuronal injury (Busto et al., 1987) such that some attenuation of injury would be expected in hibernating animals.

Several aspects of hibernation physiology are consistent with neuroprotection, including hypothermia, leukocytopenia, and other means of immune system modification, inhibition of protein synthesis, enhanced antioxidant defense, and metabolic suppression (Drew et al., 2001). Hibernation physiology emphasizes that a combination of physiological adaptations are likely necessary for the profound neuroprotection during hibernation. Neuroprotection associated with hibernation supports further research in combination therapies for stroke, head trauma and neurodegenerative disease. Multiple adaptations observed during hibernation may result from a single regulatory mechanism that could, one day, be duplicated in the clinic. An important question that remains is whether a hibernation-like state induced after trauma would be protective. Although not as appealing as complete attenuation of the traumatic tissue response, a delayed response would expand the window of opportunity for transport to medical facilities and would likely be as effective in a post-trauma clinical situation as attenuation of damage at the time of injury. Better understanding of regulatory mechanisms in mammalian hibernation may provide tools to test hypotheses regarding neuroprotective aspects of the hibernation phenotype.

Why would the myriad of potentially neuroprotective adaptations be beneficial to heterothermic mammals? One explanation may be that mechanisms of tissue response are energy demanding processes suppressed during torpor. Cold alone prevents bacteria proliferation and may provide an energy-efficient substitute for a fully responsive immune system during torpor (Zhou et al., 2002; Prendergast et al., 2002). Moreover, it is tempting to speculate that attenuation of inflammatory and apoptotic processes may protect a variety of cell types from disuse atrophy during prolonged torpor. Finally, neuroprotective adaptations may protect heterothermic animals during transitions into and out of torpor.

Better understanding of regulatory mechanisms and dynamic cellular responses has potential to explain evolutionary advantages for heterothermic adaptations and lead to improved therapies for brain-injured patients.

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δ -Opioid Agonists Protect the Rat Liver From Cold Storage and Ischemia/Reperfusion Injury

THOMAS L. HUSTED,¹ WEN-JIAN CHANG,² ALEX B. LENTSCH,¹ STEVEN M. RUDICH¹

¹ Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

² Department of Surgery, University of Michigan Health System, Ann Arbor, Michigan, USA

Abstract. δ -Opioid agonists represent a novel strategy to protect donor organs from ischemia and cold storage injuries. To determine if the synthetic δ -opioid, DPDPE ([D-Pen^{2,5}]-enkephalin) could protect the liver from damage induced by cold storage/reperfusion injury, we examined the effects of DPDPE in an *ex vivo* perfused liver rat model. Male rats were given a single intravenous injection of DPDPE or vehicle six hours prior to surgery. Following 30 minutes of global warm hepatic ischemia, the liver was removed, flushed and cold stored for six hours. Livers were then placed onto an *ex vivo* liver perfusion circuit and perfused with Krebs-Henseleit buffer for three hours. Liver function was measured by quantity and length of time of bile flow, portal vein resistance, perfusate liver function enzymes, and levels of inflammatory cytokines (TNF- δ and IL-6) in the perfusate. Rats pretreated with DPDPE exhibited significantly prolonged productive bile production compared to control animals. Pretreatment with DPDPE also protected the stressed liver from injury as the release of hepatic enzymes and pro-inflammatory cytokines were markedly reduced. The nonspecific δ -opioid receptor inhibitor BNTX completely abolished these protective effects. These data suggest that the δ_1 -opioid agonist DPDPE protects the liver from cold storage/reperfusion injury and may represent a novel treatment modality to extend the function of donor livers for transplantation.

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Introduction

The events surrounding both the cold storage of donor organs and ischemia/reperfusion (I/R) injury are of major importance in minimizing allograft dysfunction during transplantation and have particular significance as many more marginal (extended criteria donor) allografts are being used for transplantation. As such, there is a growing need to maximize the number and quality of donor organs. One possible way to increase the number of "acceptable" donor livers is to pretreat the donor (and/or the isolated liver) with organ-protective agents.

Hibernation is a unique adaptation allowing certain species, such as ground squirrels, woodchucks, brown cave bats, European hedgehogs, and black bears to survive extended periods when ambient temperatures may fall below freezing. Hibernation allows these animals to conserve up to 90% of the energy required if they were to remain active during this period. There are many parallels between hibernation and the processes involved in organ procurement and transplantation; namely ischemia, cold storage and reperfusion.

In a unique canine multiorgan block preparation in which nearly all the internal organs are removed from the animal but kept self-perfused, Chien and Oeltgen (1993) showed that perfusion with plasma from hibernating woodchucks markedly extended the viability of all the organs. This was the first evidence that moieties present in the sera of hibernating animals might have some utility to protect solid organs from ischemic damage. In a simpler animal preparation, Oeltgen et al. (1996) also demonstrated that the viability of a canine lung preservation preparation for transplantation was markedly extended when the organs were perfused with plasma from hibernating woodchucks or δ -opioid agonists.

More recently, select δ -opioid agonists have been noted to confer organo-protective effects. Two of these agonists are the nonspecific δ -opioid receptor agonist DADLE ([D-Ala²-Leu⁵¹]-enkephalin) and the specific δ_1 -opioid receptor agonist DPDPE ([D-Pen^{2,5}]-enkephalin). Bolling et al. (1997) demonstrated in a rabbit isolated cardiac perfusion model that perfusate containing DADLE significantly prolonged preservation time and improved cardiac dynamics, histology, and preserved ultrastructure, as compared to a control preparation not containing such peptides. In another rabbit model, Tubbs et al. (2002) have shown that DADLE can impart ischemic tolerance to the isolated jejunum. Most recently, Yamanouchi et al. (2003) have shown DADLE to protect against warm ischemia reperfusion (I/R) injury in rat liver. In the current studies, we sought to determine if synthetic specific δ_1 -opioid agonist, DPDPE, could protect the rat liver from cold storage and reperfusion injury. Furthermore, to assess whether

the effects of DPDPE were linked to the δ -opioid receptor, experiments were also performed with the nonspecific δ -opioid receptor inhibitor 7-benzylidene naltrexamine (BNTX).

Methods

Male Sprague-Dawley rats weighing 225–275 grams were used in all experiments, being kept on a 12-hour wake/sleep cycle with food being withheld 12 hours prior to surgery. Rats were injected via the tail vein with either 0.2 mL of δ_1 -opioid agonist, [D-Pen^{2,5}]-enkephalin (DPDPE) at 2 mg/kg animal weight or saline vehicle, six hours prior to surgery. After 6 hours, the animals were anesthetized with phenobarbital (intra-peritoneal) and placed onto a heated operating table. Following midline laparotomy, 30 minutes of global hepatic ischemia was imposed by placing a micro-clip across the porta hepatis. The bile duct, infra-hepatic vena cava and supra-hepatic vena cava were then cannulated. Following sacrifice, the liver was flushed with cold University of Wisconsin (UW) solution until the effluent from the supra-hepatic vena cava was clear. The liver was then immersed in UW solution and kept stored on ice for 6 hours until *ex vivo* hepatic perfusion. In select experiments, the δ -opioid receptor antagonist BNTX (0.5 mg/kg) was injected via the tail vein 30 minutes prior to injection with the study agent. Between five and eight animals were used for each experiment.

Livers stored for six hours were placed onto a custom-designed ex-vivo hepatic perfusion apparatus (Hugo Sachs Elektronik, Freiburg, Germany) (Fig. 1). This device, temperature-controlled in an enclosed box, consists of a membrane oxygenator, constant pressure pump, flow and pressure sensors at both the inflow (portal vein) and outflow (supra-hepatic vena cava), as well as on-line oxygen, potassium, and pH sensors. The perfusate (250 ml in total volume) was a modified Krebs-Henseleit buffer, kept at 37° C. During each perfusion experiment, samples were taken from the perfusate, bile, and liver for analysis. Samples were kept cold until just prior to analysis. Perfusion was performed until no further bile was produced or for three hours, whichever was longer. Bile was collected and measured every 15 minutes during the perfusion. At the termination of each experiment, 2 gm samples of liver were taken, blotted dry, and placed into an oven (80° C). Wet/dry weight ratios were calculated after 48 hours in the oven. Samples of liver tissue, taken both during and at the termination of each experiment, were placed into neutral-buffered formalin for H & E as well as TUNEL staining, using standard techniques.

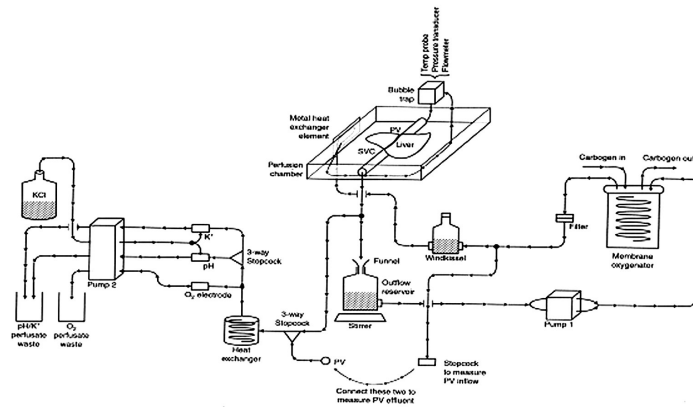


Fig. 1. Ex vivo rat liver perfusion apparatus, containing pressure transducers and in-line sensors to measure oxygen, pH, and potassium. The entire device is enclosed in a constant temperature chamber.

Measurements of AST, ALT, LDH, total bilirubin, and potassium were performed in the clinical laboratory using automated analyzers. In addition, perfusate pH and potassium levels were monitored using in-line sensors, both of these giving a real-time indication of the viability of the perfused liver. Cytokine analyses, including TNF- α and IL-6, were performed using standard ELISA kits and manufacturer's instructions.

Comparisons between treatment groups were performed using a Student's *t*-test, with a *p* value of 0.05 being the cut-off for significance. For clarity, error bars were not incorporated into the figures. The standard deviation of the means shown in the figures were not greater than 20%.

Results

As a marker of hepatic function, bile production was measured from the *ex vivo* perfused liver. In livers from rats pretreated with saline, bile production peaked at 60 minutes and then showed a slow decline. However, livers pretreated with DPDPE had significantly greater bile production, both in terms of length and quantity, compared to saline pretreatment ($p < 0.002$). Concurrent pretreatment with BNTX abrogated the beneficial effects of DPDPE, yielding bile production similar to saline pretreated livers (Fig. 2).

A measure of endothelial damage and organ congestion is portal vein resistance (PVR). As the liver is damaged, the sinusoidal endothelial cells become

edematous, and encroach upon the Space of Disse, leading to increased resistance of fluid moving through the liver. PVR was elevated in saline pretreated livers (0.35 mmHg/mL/min) and continued to rise over the length of perfusion. DPDPE pretreated livers showed a decreased PVR throughout the entire three hours of perfusion with a nadir at 0.21 mmHg/mL/min at 1.5 hours (Fig. 3). Pretreatment with DPDPE and BNTX reflected similar PVR as saline pretreated samples ($p < 0.05$). As another measure of hepatic edema, hepatic tissue wet/dry weight ratio was obtained (data not shown) at the completion of perfusion. DPDPE treated livers maintained the lowest ratio (3.75 ± 0.56) compared to saline (4.35 ± 0.34) or pretreatment with DPDPE and BNTX (4.3 ± 0.45). These differences did not reach statistical significance.

As a measure of hepatocyte injury, liver function enzymes were assessed from the perfusate. Saline pretreated livers showed elevated enzyme release following

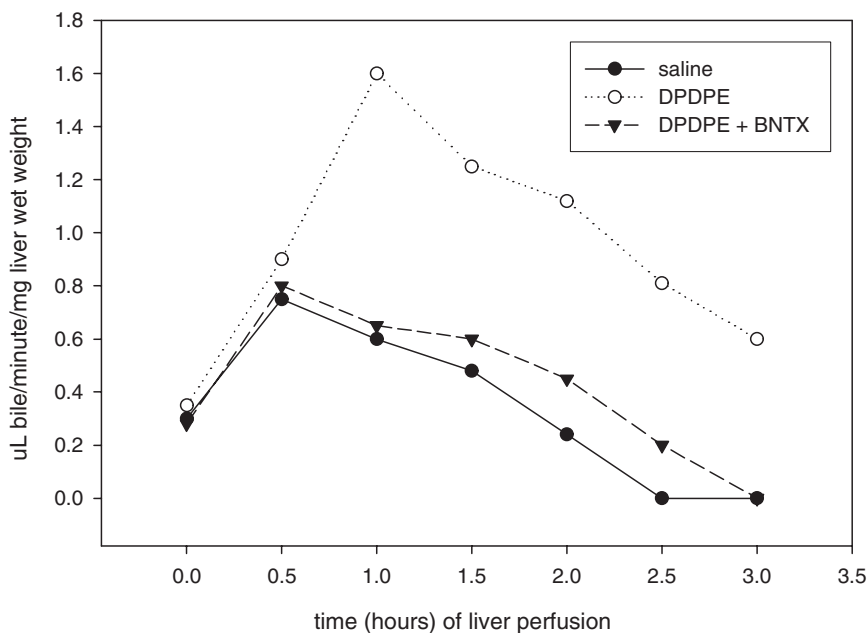


Fig. 2. Bile production (mg bile/min/mg tissue) of rat livers during ex vivo perfusion. Pretreatment with DPDPE greatly increased volume and duration of bile production. BNTX treatment abolished the effects of DPDPE. Values represent mean \pm SEM. In all figures, between five to eight rodents were used for each experiment. $P < 0.002$ compared to both saline and BNTX pretreatment.

30 minutes of global warm ischemia and six hours of cold storage. Pretreatment with DPDPE significantly reduced enzyme release, to levels nearly four-fold less than untreated animals, $p < 0.002$. (Fig. 4). Pretreatment with DPDPE and BNTX revealed AST, ALT, and LDH levels similar to saline pretreatment. In addition, there was less potassium released from the DPDPE treated livers (3.4 ± 0.7 mEq/mL) compared to saline treatment (4.5 ± 0.9 mEq/mL) or DPDPE with BNTX (4.3 ± 0.8 mEq/mL) (data not shown).

Proinflammatory cytokine release was measured at 60, 90, and 150 minutes after reperfusion on the *ex vivo* perfusate device. Elevated concentrations of the proinflammatory cytokines TNF- α and IL-6 were measured for saline pretreated livers, peaking at 90 minutes. DPDPE pretreated livers had significantly less secretion of TNF- α and IL-6, peaking at 90 minutes (TNF- α 45pg/mL, IL-6:

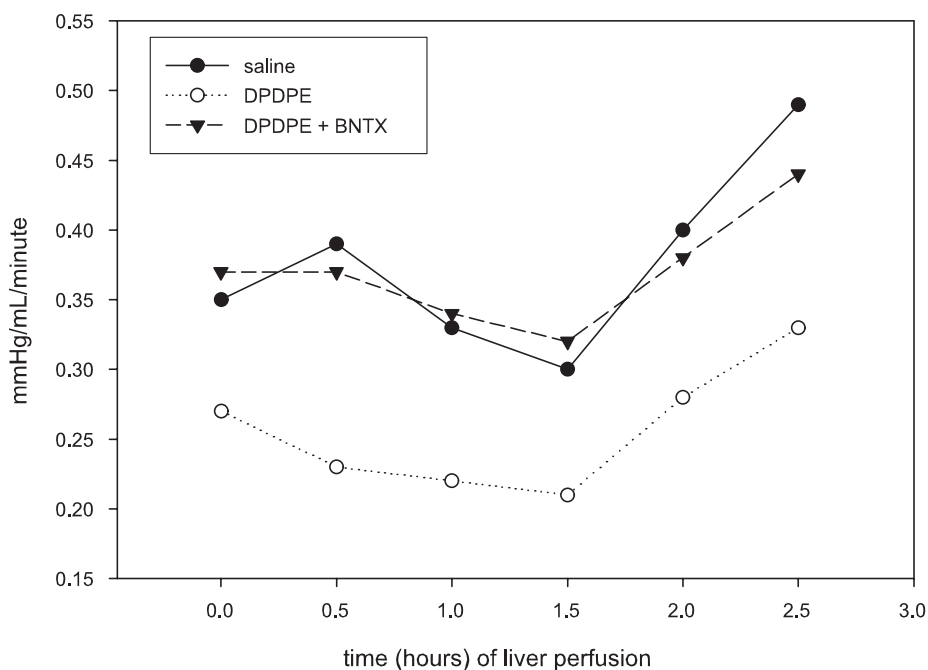


Fig. 3. Portal vein resistance (mm Hg/ml/min) of rat livers during *ex vivo* perfusion. Pretreatment with DPDPE resulted in a marked decrease in resistance, while BNTX abolished this effect. This suggests that livers treated with DPDPE had less edema and sinusoid swelling as compared to saline controls. $P < 0.05$ compared to saline and BNTX pretreatment.

38 pg/mL). Concurrent pretreatment with DPDPE and BNTX resulted in cytokine release similar to saline pretreatment and approximately twice that of DPDPE treated livers ($p < 0.02$) (Fig. 5a and b). The decrease in cytokine secretion was over three-fold insofar as TNF- α secretion, whereas it was only two-fold for IL-6.

Histologic examination of the livers at the termination of the perfusions was performed using both H&E and TUNEL staining (data not shown). H&E staining showed significant disruption of the normal hepatic parenchyma in the saline treated and DPDPE with BNTX treated livers, while the DPDPE-treated livers had nearly normal hepatic architecture. Additionally, TUNEL staining revealed a marked degree of apoptosis (up to 80%) in the saline-treated control liver compared to less than 15% apoptosis observed in DPDPE-treated rodents.

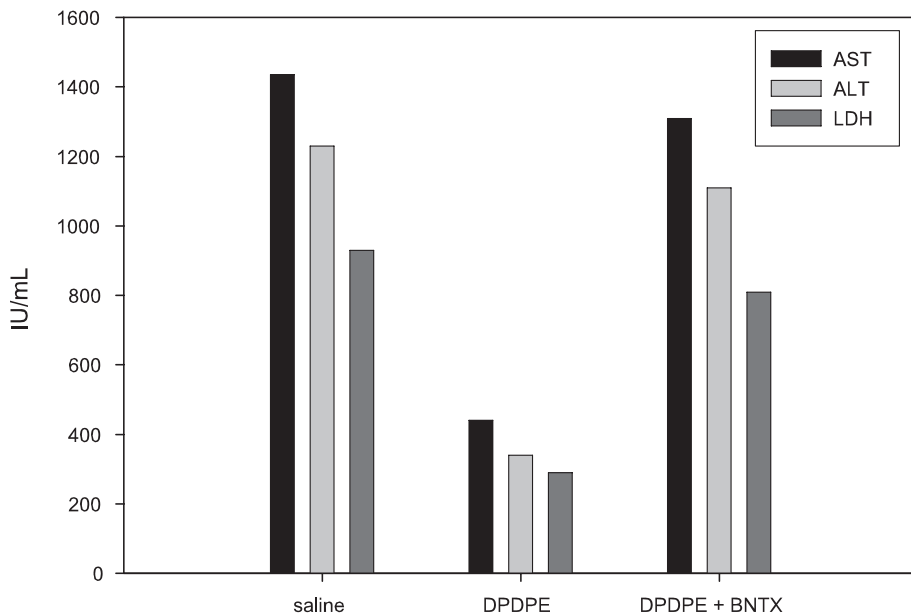


Fig. 4. Perfusate transaminase levels (IU/mL) during ex vivo perfusion. Pretreatment with DPDPE resulted in significant reduction in AST, ALT, and LDH levels. BNTX abrogated these protective effects on hepatocyte viability. Values represent mean \pm SEM. $P < 0.02$ compared to saline and BNTX pretreatment.

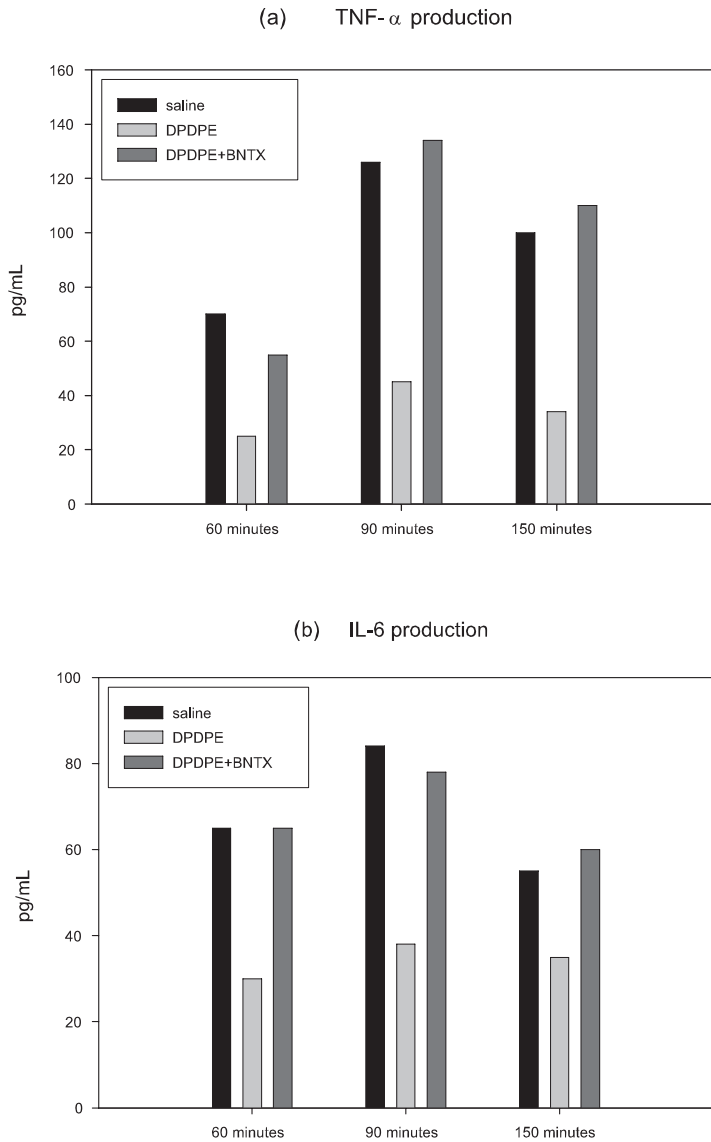


Fig. 5. Proinflammatory cytokine release (pg/ml) measured from perfusates of rat livers during ex vivo perfusion. Pretreatment with DPDPE resulted in significantly less (a) $\text{TNF-}\alpha$ and (b) IL-6 release than saline control or BNTX blocked livers. Maximum cytokine release was observed after 90 minutes of perfusion. Values represent mean \pm SEM. $P < 0.02$ compared to saline pretreatment.

Discussion

Our studies provide evidence that δ_1 -opioid agonists, such as DPDPE, may be highly useful in the field of transplantation. In our rat model of cold storage/reperfusion injury, pretreatment with DPDPE greatly augmented liver function as measured by bile production and portal vein resistance. Treatment with DPDPE also reduced biochemical and histological parameters of liver injury, demonstrating that this peptide (and presumably other members of this enkephalin family) not only helped to preserve liver function, but also prevented ischemia/reperfusion-induced injury. Due to the use of a buffer, instead of whole blood as the perfusate, it is likely that much, if not all of the injury to the liver, was a result of oxidant stress. Thus, DPDPE may render hepatocytes more resistant to oxidant-induced injury.

Treatment with DPDPE also greatly attenuated the elaboration of the pro-inflammatory cytokines, TNF- α and IL-6. TNF- α , produced primarily by the resident liver macrophage population (Kupffer cells), is known to be a major component of the inflammatory response to ischemia/reperfusion injury in the liver. Thus, our data also provide evidence that DPDPE may suppress the activation of Kupffer cells and their production of TNF- α .

This work builds on that of Bolling, and is the first experimental evidence to show that small molecule peptides (in the form of opioid agonists) can have a pronounced effect on liver injury, in a model that mimics many of the events in solid-organ transplantation. Although no direct correlation exists between hibernation and the δ -opioid agonists, it has been long known that hibernation is accompanied by changes in the brain opioid system. Perhaps by taking advantage of the binding of opioid receptors in nonhibernators, we can "mimic" the biologic adaptation which hibernators express to protect their vital internal organs during extended periods of torpor.

In summary, the current studies provide strong evidence to support the use of δ_1 -opioid agonists, such as DPDPE, for pre-conditioning donor livers for transplantation. Further examination of the molecular mechanism by which these agonists confer protection in different liver cell types may provide important information allowing their use in the clinical setting.

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Animal Adaptability to Oxidative Stress: Gastropod Estivation and Mammalian Hibernation

MARCELO HERMES-LIMA,¹ GABRIELLA R. RAMOS-VASCONCELOS,¹ LUCIANO A. CARDOSO,¹ ADRIENNE L. ORR,² PATRICIA M. RIVERA,² AND KELLY L. DREW²

¹ Universidade de Brasília, Departamento de Biologia Celular, Brasília, 70910-900, Brazil

² University of Alaska Fairbanks, Institute of Arctic Biology, Fairbanks, AK 99775, USA

Abstract: We have discussed the role of antioxidant defenses (antioxidant enzymes, ascorbate, and glutathione) in the protection of estivating gastropods and hibernating ground squirrels from specific conditions where reactive oxygen species (ROS) may be overproduced. Such conditions of potential oxidative stress of physiological nature are compared with those in ectothermic vertebrates that endure wild cycles of oxygen availability, such as during anoxia endurance (followed by reoxygenation, when ROS can be overproduced).

I. ROS and Antioxidants in Animals Living in Extreme Conditions

Animals use many strategies to survive the seasonal changes in the environment, which are related to temperature (from hot to very cold/freezing), humidity, food and water availability, salinity, and oxygen concentration. Some changes in the environment can be extremely drastic when, for example (i), oxygen supply to ice-covered water bodies is too low to maintain aerobic metabolism in fish and in frogs or turtles hibernating in the bottom of those water bodies (Storey, 1996; Lushchak et al., 2001). Survival under anoxia for periods of weeks is a key adaptation for many invertebrates as well. Other conditions include survival (ii) when over 40–50% extracellular water freezes in overwintering frogs, snakes and hatchling turtles (causing arrest of circulation and ischemia to internal organs; Storey, 1996); (iii) when there is over 30–40% dehydration in toads and frogs

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(causing strong reduction in blood flow and hypoxia to internal organs), either in very cold climates or in desert conditions (Hermes-Lima and Zenteno-Savín, 2002); (iv) when water and food availability is so low that snails estivate in their shells and desert toads burrow underground under estivation (Hermes-Lima et al., 1998); (v) when diving in aquatic reptiles and mammals is so extended that internal organs become severely hypoxic (Hermes-Lima and Zenteno-Savín, 2002); or (vi) during mammalian hibernation (see below).

The integrated study of the behavioral, physiological, and metabolic adjustments of animals under those conditions has been the subject of research for the past several decades. However, the study of the involvement of free radicals and antioxidants in the processes of adaptation to these stresses has only started in the 1990s. In the conditions when oxygen availability is reduced or even cut off (during freezing, dehydration, anoxia/hypoxia exposure, and long apneic dives), internal organs of these animals have to cope with a situation very much like ischemia in “regular” mammals. With ischemia, oxygen and energetic supply (e.g., glucose delivery) is greatly decreased, forcing cells to endure a potentially fatal energy deficit. The reoxygenation of these cells creates another potentially damaging event of overproduction of reactive oxygen species (ROS), inducing cellular oxidative damage (Lipton, 1999; Hermes-Lima, 2004). Even though ischemia/reperfusion can be disastrous to human organs, many animals experience this situation in the wild with extraordinary survival rate (Hermes-Lima and Storey, 1996; Storey, 1996).

The search for how animals cope with these situations of potential oxidative stress has started with studies of internal organs of garter snakes *Thamnophis sirtalis parietalis* under either anoxia (10 hours, 5° C) or freezing exposure (5 hours, -2.5° C), and with the brain of anoxia-tolerant turtles. In the case of garter snakes, Hermes-Lima and Storey (1993) showed that some snake organs increase the activity of certain antioxidants during anoxia (total-superoxide dismutase activity [total-SOD], and glutathione [GSH] levels) or freezing (selenium-dependent glutathione peroxidase [Se-GPX] and catalase) as a form of preparation for the potential oxidative stress caused by reoxygenation or thawing. In the case of turtles, studies from Rice's laboratory showed that a high constitutive level of ascorbate (but not of GSH) is associated with tolerance to hypoxia and reoxygenation stress (Rice et al., 1995).

Other studies showed that antioxidant defenses are also of relevance against the stress of oxygen reperfusion in goldfish *Carassius auratus*, red-eared turtles *Trachemys scripta elegans*, leopard frogs *Rana pipiens*, and marine gastropods

Littorina littorea under anoxia exposure (8 hours, 20 hours, 30 hours, and 6 days, respectively) (Hermes-Lima and Zenteno-Savín, 2002). The role of antioxidant enzymes and/or GSH in the form of anticipatory defense was also demonstrated in leopard frogs under severe dehydration (50% loss of body water in 4 days, 5° C) and in wood frogs *Rana sylvatica* under 24 h freezing at -2.5° C (Hermes-Lima and Zenteno-Savín, 2002). The common biochemical adaptation of these animals (except in turtles) is the “preparation for oxidative stress,” which is the build-up of antioxidant defenses while ROS are minimally produced. Red-eared turtles maintain high constitutive levels of antioxidant defenses during anoxia, even though some enzymes secondarily involved in antioxidant defense (such as GSH synthetase) are increased in a few organs (Hermes-Lima and Zenteno-Savín, 2002).

2. Physiological Oxidative Stress and Estivation in Snails

We have also been interested in the role of antioxidant defenses in the estivation process of land snails. During estivation there is a drop in oxygen tension in internal organs, and metabolic rates can go as low as 5% of the active state (this is species-specific). However, snails maintain a minimal level of aerobic metabolism during estivation (Storey and Storey, 2004). When food and/or water are available land snail species return to the active state. In some cases the arousal process is quick and in several minutes snails are fully active again. This transition from estivation to the active state, the arousal period, has been shown to involve a pronounced and transitory increase in oxygen uptake, which is higher than the normal active state. We have proposed that arousal in land snails could result in oxidative stress due to the increased oxygen uptake—which may increase the rates of mitochondrial ROS formation (Hermes-Lima et al., 1998).

Indeed, we observed that during awakening there is a transitory increase in lipid peroxidation, measured as TBARS, in the hepatopancreas of land snails *Otala lactea* (arousal from 30-day estivation; Hermes-Lima and Storey, 1995) and *Helix aspersa* (arousal from 20-day estivation; Ramos-Vasconcelos and Hermes-Lima, 2003). In the case of *H. aspersa* hepatopancreas, there is also a transitory increase (lasting several minutes) in the ratio between glutathione disulfide (GSSG) and GSH-equivalents ($\text{GSH-eq} = \text{GSH} + 2 \text{GSSG}$); this is indirect evidence of increased detoxification of peroxides during arousal. In both land snail species, protection against oxidative stress in hepatopancreas (upon arousal from estivation) may be due to higher activity of specific antioxidant enzymes during estivation: Se-GPX and total-SOD in *O. lactea* and Se-GPX

(see Table 1) in *H. aspersa*. In foot muscle, only Se-GPX activity was increased in *H. aspersa* (Table 1), while total-SOD, catalase, and glutathione S-transferase (GST) activities were significantly increased in *O. lactea*.

In the case of *H. aspersa* and *O. lactea*, the increase in the activity of specific antioxidant enzymes was accompanied by unchanged activity of others. For example, while foot muscle Se-GPX was increased (Table 1) in *H. aspersa*, the activities of total-SOD, catalase, GST, and glutathione reductase (GR) were unchanged. However, this was not true for the case of estivating *Biomphalaria tenagophila*, a freshwater snail. After 15 days of estivation, Se-GPX activity increased significantly (by 14%) in hepatopancreas, while total-SOD activity dropped by 43%; catalase, GST and GR activities were unchanged (Ferreira et al., 2003). It is interesting that 24 h underwater anoxia exposure in *B. tenagophila* also induced an increase in Se-GPX activity (by 40%) in hepatopancreas, while catalase activity dropped by 31%. The augment in Se-GPX activity during estivation and anoxia exposure in *B. tenagophila* may be a relevant event for the management of ROS formation when metabolic rate is restored upon arousal and reoxygenation (Ferreira et al., 2003).

3. Summer Versus Winter Estivation in Land Snails

The estivation observations by Ramos-Vasconcelos and Hermes-Lima (2003) with *H. aspersa* were all carried out in the winter season in Brasília (midwestern Brazil, South America). We also observed that summer estivation in *H. aspersa* (in the same laboratory conditions as in winter) brought about an increase in Se-GPX activity (see Table 1) in hepatopancreas. However, catalase and total-SOD activities were significantly diminished during summer-estivation (Ramos,

Table 1: Se-GPX activity (mUnits/mg protein) in *Helix aspersa* under 20-day estivation in summer or winter, and 24 h in the post-arousal active state.

	Winter snails		Summer snails	
	Estivating	Active	Estivating	Active
Hepatopancreas	26.5 ± 5.4 (4) ^a	5.4 ± 2.1 (5)	161.0 ± 39.3 (12) ^a	43.8 ± 9.0 (12)
Foot-muscle	12.5 ± 2.3 (5) ^a	4.2 ± 0.7 (5)	9.1 ± 1.5 (11)	6.9 ± 1.0 (12)

Data from Ramos (1999) and Ramos-Vasconcelos and Hermes-Lima (2003), represented as mean ± SEM (with n values).

^a Significantly different from active snails, $P < 0.01$.

1999), which might be a side effect of hypometabolism on protein biosynthesis. No changes in antioxidant enzymes (including Se-GPX; Table 1) were observed in foot muscle in summer estivation. In the case of GSH-eq, its hepatopancreas concentration was 1.8-fold higher in winter estivation ($2.9 \mu\text{mol/g}$ wet wt.) in comparison with 24-h awake snails (Ramos-Vasconcelos and Hermes-Lima, 2003); in summer, hepatopancreas GSH-eq concentration was unchanged (Cardoso LA, unpublished). These data indicate that seasonality has a relevant influence in the regulation of the antioxidant apparatus of estivating land snails.

4. Is Oxidative Stress Associated With Hibernation in Heterothermic Mammals?

Hibernating mammals provide another example of adaptation to extreme environments where enhanced antioxidant defense might play a cytoprotective role. Physiologists from Belgrade were the first to link - back in 1990 - mammalian hibernation with season and hibernation-associated increases in antioxidants. In interscapular brown adipose tissue (BAT) of hibernating ground squirrels (*Citellus citellus*), they observed significant increases in the activities of GPX and total-SOD, and in ascorbate concentration, when compared with active animals from spring and autumn (Buzadzic et al., 1990). GPX also increased in the liver, as did ascorbate in the plasma. Such an increase in the antioxidant capacity of interscapular BAT was postulated to be protective against ROS generation resulting from the intense metabolic activity of this tissue in periodic arousals during the course of hibernation (Buzadzic et al., 1990). The Belgrade authors continued working in the following years with *C. citellus* to study seasonal changes in antioxidant defense mechanisms (e.g., Blagojevic et al., 1998; Buzadzic et al., 1998). However, these authors did not determine whether or not there is oxidative stress associated with hibernation/awakening.

More recently, Alaskan workers and associates became interested in free radical metabolism in arctic ground squirrels *Spermophilus parryii* and thirteen-lined ground squirrels *Spermophilus tridecemlineatus*. During the eight-month hibernation season, oxygen consumption falls to 2% of basal levels. It then rises to 300% of hibernating levels during periodic arousals (Drew et al., 2002b), which happens once every one to two weeks for periods of approximately 24 h. Body temperature increases from 2 to 37° C during the two to three hour period of awakening. Drew et al. (1999) observed a significant two-fold increase in ascorbate concentration in the cerebral spinal fluid of *S. parryii* and an increase in plasma ascorbate (by three-to-four fold) in both *S. parryii* and *S. tridecemlin-*

eatus during hibernation. Plasma ascorbate then falls to euthermic values at the time of peak oxygen consumption and cerebral blood flow (Tøien et al., 2001). These data suggest that elevated concentrations of ascorbate in plasma and CSF may protect hibernating ground squirrels, specifically their neurons (Drew et al., 2002b), from oxidative damage during either hibernation or arousal. The transient increase in plasma uric acid, a product from xanthine oxidase-catalyzed reaction (which also produces O_2^- and H_2O_2), during arousal of *S. parryii* was considered indirect evidence for excess ROS generation resulting from the dramatic augment in metabolic rate (Tøien et al., 2001; Drew et al., 2002b).

Although evidence suggests antioxidant defense mechanisms increase seasonally as well as during hibernation, few studies have monitored oxidative stress during hibernation or following arousal. We have recently assessed oxidative stress in BAT from winter-euthermic, winter-hibernating, and three-hour aroused *S. parryii* and observed no significant changes in lipid peroxidation (as TBARS) or protein oxidation, as carbonyl protein (Orr et al., 2003). However, GSH-eq concentration in BAT of euthermic squirrels (about 1.5 $\mu\text{mol/g}$ wet wt.) was 1.5-fold higher than in hibernating animals but not different from aroused ones. Levels of GSH were also higher (by 1.7-fold) in BAT of euthermic animals when compared with hibernating ones; GSSG levels were unchanged. An apparent increase in the GSSG/GSH-eq ratio was seen during hibernation, but this was not statistically relevant (Orr et al., 2003). These data suggest that ROS generation in BAT during hibernation and arousal is not causing oxidative stress, possibly due to tight control by antioxidant defenses.

On the other hand, an increase in lipid peroxidation (measured as conjugated dienes, CD) in the intestinal mucosa of thirteen-lined ground squirrels *S. tri-decemlineatus* was observed in Carey's laboratory in winter-hibernating animals in comparison with summer-active (euthermic) ones; arousing animals (from winter) also presented diminished CD levels in comparison with squirrels under short-term winter torpor (Carey et al., 2000). Moreover, the GSSG/GSH ratio increased 3-to-5 fold in intestinal mucosa from squirrels in all hibernation states during the winter (including torpid, arousing, and interbout arousal) when compared with summer-euthermic squirrels. Such an effect was possibly due to a 50% decrease in GR activity, causing concomitant increase in GSSG (Carey et al., 2003). Furthermore, GSH-eq from intestinal mucosa increased during interbout arousal and remained elevated throughout entrance and early torpor compared to summer-active animals (Carey et al., 2003). Another study compared blood samples from three female black bears collected in the active state (early

fall) and under winter hibernation. There was a significant increase in TBARS in plasma and in RBC membranes from hibernating bears (Chauhan et al., 2002). These studies (with squirrels and bears) suggested that hibernation is associated with oxidative stress and may also reflect tissue differences. For instance, gut may be vulnerable to absence of nutrient intake and RBC, lacking mitochondria, may be vulnerable to decreased glucose supply during hibernation.

Even though the overall observations amassed so far are still fragmented (several different animal models with few tissues analyzed), there is a general thought that hibernation/arousal is linked with oxidative stress of physiological nature and/or enhanced antioxidant defenses. However, it is imperative to study other tissues and conduct experiments comparing hibernating with euthermic and arousing animals in the same season, under similar environmental conditions (seasonality itself can be a confounding factor since it affects free radical metabolism; see topic 3). Time points of tissue collection and control conditions must be carefully chosen, depending on the question asked. For example, mitochondrial ROS production would be expected to be maximal during peak O_2 consumption, while later time points might reflect a balance of accumulated damage and detoxification/repair. In contrast to estivating snails, organs of hibernating squirrels (and bears) are not under hypoxia, and thus oxygen is not limited for ROS production (even though mitochondrial respiration is repressed and subsequent ROS production should be minimal). Furthermore, recent evidence in bats and arctic ground squirrels suggest that internal organs of these animals are hypoxic during arousal (Drew et al., 2002a). More work is required to place this new evidence into the hibernation puzzle.

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